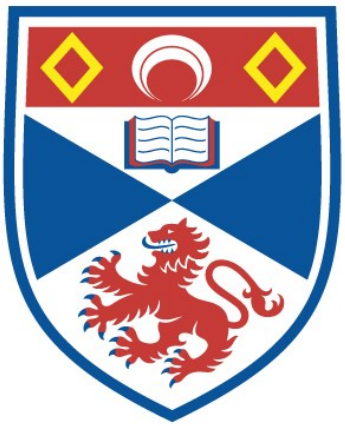
THE DETECTION AND IDENTIFICATION BY ELECTRON MICROSCOPY OF A POLYOMAVIRUS (FRKV) CONTAMINATING FETAL RHESUS MONKEY KIDNEY CELL LINES USED TO GROW HEPATITIS A VIRUS

Joan Elizabeth Richmond

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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THE DETECTION AND IDENTIFICATION BY ELECTRON MICROSCOPY OF A POLYOMAVIRUS (FRKV) CONTAMINATING FETAL RHESUS MONKEY KIDNEY CELL LINES USED TO GROW HEPATITIS A VIRUS

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A thesis submitted to the University of St Andrews

for the degree of Doctor of Philosophy

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ABSTRACT

A continuous line of fetal rhesus monkey kidney cells, FRhK-4, has been used for the propagation of hepatitis A virus (HAV) to prepare diagnostic antigen. While studying the fine structure of HAV grown in these cells, I discovered an unsuspected polyomavirus in both HAV-inoculated and control, uninoculated cultures.

The virus, designated FRKV, was also detected in low pass FRhK-4 cultures and in FRhK-6, a cell line used in the development of a live, attenuated hepatitis A vaccine. The presence of an adventitious polyomavirus in FRhK-6 cultures poses questions about the suitability of these cells for vaccine preparation.

I have used immunoelectron microscopy (IEM) to identify FRKV, which is not antigenically related to the primate polyomaviruses SV40, SA12, BKV or JCV, but reacts with antibodies that are present in fetal, newborn and adult bovine sera. By IEM the virus is indistinguishable from WRSV, a polyomavirus isolated from calf kidney. FRKV is antigenically similar to recently isolated calf kidney polyomaviruses. I have demonstrated that a commercial pool of fetal bovine serum contains infectious virus and its antibody. FRKV is therefore almost certainly a bovine polyomavirus.

IEM has also shown that FRKV is indistinguishable from STMV and CMKV, the polyomaviruses isolated from cultures of stump-tailed and cynomolgus macaque kidney, and is antigenically similar to a polyomavirus from another monkey kidney cell line, LLC-MK2. By implication FRKV is similar to HD, the STMV-like polyomavirus from Vero cells.

It thus appears that various monkey kidney cultures from several laboratories have been contaminated with a bovine polyomavirus. It follows that highly sensitive techniques are required for screening bovine sera used in culture media, especially those used in vaccine production. I have explored the use of antibody-coated grids to increase the sensitivity of EM but conclude that the greater sensitivity of DNA amplification techniques might be more appropriate for this purpose.

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DECLARATION

I, Joan Elizabeth Richmond, hereby certify that this thesis has been composed by myself, that it is a record of my own work and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

I was admitted to the Faculty of Science of the University of St Andrews under Ordinance General No 12 in June 1981 and as a candidate for the degree of PhD in October 1982.

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of PhD.

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I am most grateful to Dr P P Mortimer for his encouragement and constructive criticism of the manuscript. I am also grateful to Mrs E C Paddon who printed the proofs of many of the electron micrographs, to Mr A A Porter who did much to ensure the smooth running of the electron microscopes and the PHLS Library staff for advice and assistance in obtaining copies of several publications.

Finally, I would like to thank my husband, Bill, whose constant encouragement and loving support made this work possible.

ABBREVIATIONS

ACG	antibody-coated grids
BDV	bovine diarrhoea virus
BFDV	budgerigar fledgling disease (polyoma)virus
BHK-21	baby hamster kidney cell line
BKV	human polyomavirus BK
BPA	bovine plasma albumin
BSC-1	African green monkey kidney cell line
CCL 33	swine kidney cell line
CCL 33 PV	swine polyomavirus isolated from CCL 33 cell line = SPV
CIE	counter-immunoelectrophoresis
CK culture	calf kidney culture
CKnV	calf kidney virus from CKn culture eg CK8V from CK8 cells
CKV	calf kidney (polyoma)virus
CMKV	cynomolgus macaque kidney (polyoma)virus
CPE	cytopathic effect(s)
CR326	strain of HAV isolated from serum, CR326F from faeces
DBS-FRhL-2	Division of Biologic Standards fetal rhesus lung cell lines
DNA	deoxyribonucleic acid
ECHO	enteric cytopathic human orphan (picornaviruses)
EM	electron microscopy
ER	endoplasmic reticulum
FBS	fetal bovine serum
FPV	frog papova-like virus
FRhK-4	fetal rhesus kidney 4 culture
FRhK-6	fetal rhesus kidney 6 culture
FRK4V	polyomavirus from FRhK-4 culture
FRK6V	polyomavirus from FRhK-6 culture
FRKV	fetal rhesus kidney (polyoma)virus
GAB	goat anti-bovine
GM	growth medium
H antigen	heat-treated poliovirus antigen
HAAg	hepatitis A antigen
HAV	hepatitis A virus
HBsAg	hepatitis B surface antigen
HD	STMV-like polyomavirus from Vero cells
HEK	human embryo kidney

HPV	hamster polyomavirus - relationship to LHV unknown
IEM	immunoelectron microscopy
IF	immunofluorescence
IgG, IgM	immunoglobulin G, immunoglobulin M
JCV	human polyomavirus JC
K	a murine polyomavirus not related to polyoma
LHV	latent hamster (polyoma)virus - relationship to HPV unknown
LLC-MK2	rhesus monkey kidney cell line
LLC-MK2V	polyomavirus isolated from LLC-MK2 cell line
LPV	lymphotropic polyomavirus
M-PML	polyomavirus in PML-like lesions in macaque brain
MA104	rhesus monkey kidney cell line
MA104V	polyomavirus isolated from MA104 cell line
MDBK	Madin Darby bovine kidney cell line
MEM	minimum essential medium
MM	maintenance medium
MRC-5	human diploid cell line
MS-1	strain of hepatitis A virus
MV	Mengo virus
N antigen	native poliovirus antigen
NBS	newborn bovine serum
OMKV	owl monkey kidney (polyoma)virus
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PK-15	pig kidney cell line
PML	progressive multifocal leukoencephalopathy
polyoma	mouse polyoma virus
PP-2	*Polyomavirus papionis 2* - baboon virus not related to SA12
PTA	phosphotungstic acid
RAB	rabbit anti-bovine
RDE	receptor destroying enzyme
RER	rough endoplasmic reticulum
RIA	radioimmunoassay
RKV	rabbit kidney vacuolating (polyoma)virus
RNA	ribonucleic acid
RPV	rat polyomavirus
RS	respiratory syncytial
RT	room temperature

SA12	simian agent 12 - baboon polyomavirus
SPIEM	solid phase immunoelectron microscopy
SPV	swine polyomavirus
SRP	small round(ish) particles
STMV	stump-tailed macaque (polyoma)virus
SV40	simian virus 40 - rhesus monkey polyomavirus
SV40-PML	SV40 isolated from patients with PML
T antigen	tumour antigen
TMV	tobacco mosaic virus
UA	uranyl acetate
Vero	African green monkey kidney cell line
WI-38	human diploid cell line
WRSV	Wokalup Research Station virus - calf polyomavirus

1. INTRODUCTION

1.1. Discovery of fetal rhesus kidney virus - FRKV

In this thesis I illustrate, in a series of electron micrographs and tables summarizing electron microscopy (EM) experiments, the discovery of an adventitious polyomavirus, designated FRKV, in FRhK-4 cultures.

Wallace and her co-workers (1973 a & b) established several cell lines from fetal rhesus monkey tissues including kidney. Two of these lines, FRhK-6 and FRhK-4 (Provost & Hilleman, 1979; Flehmig, 1980), have been used to grow hepatitis A virus (HAV). The FRhK-4 cell line was originally obtained in order to investigate its suitability for production of HAV antigen as a diagnostic reagent. During a study of HAV morphogenesis in these cells, I unexpectedly observed polyomavirus particles in negatively stained preparations of fluids from HAV-inoculated and uninoculated FRhK-4 cultures.

To determine whether or not this virus was a recent contaminant, lower passage FRhK-4 and FRhK-6 cells were obtained from the Bureau of Biologics, Maryland, USA, which holds the original cell stocks; FRKV was present in these cultures after prolonged incubation. The virus has also been detected in uninoculated DBS-FRhL-2 cells and calf kidney (CK) cultures. It is present in LLC-MK2 cells from another laboratory.

1.2. Human hepatitis A virus - HAV

Human HAV defied isolation for more than half a century (Hersey & Shaw, 1968) before Deinhardt and his colleagues (1967) provided evidence for propagation in marmosets. However, it was not until 1973 that Feinstone, Kapikian and Purcell demonstrated by EM the presence of spherical 27 nm particles in stools collected from patients during the acute phase of hepatitis A infection. A serological response to the

virus antigen was demonstrated in the patients confirming that the particle was the aetiologic agent of hepatitis A (Feinstone et al, 1973). Strain CR326 of human HAV, purified from infected marmoset sera and liver extracts, gave specific reactions with human sera containing anti-HAV and antibody coating of the 27 nm virions was demonstrated by immunoelectron microscopy (IEM) (Provost et al, 1975).

After several passes in marmosets, *Sanguinus mystax* and *Sanguinus labiatus*, strain CR326 grew well in FRhK-6 cells, without producing a cytopathic effect (CPE). This cell line was the first reliable substrate for propagation of human HAV *in vitro* for diagnostic antigen preparation and vaccine production (Provost & Hilleman, 1979).

An experimental live attenuated HAV vaccine was studied in marmosets and chimpanzees and this was followed by trials in human volunteers (Provost et al, 1986). The seed HAV used in the preparation of this vaccine had been passaged 15 times in FRhK-6 cells before being adapted to grow in human diploid lung fibroblast (MRC-5) cultures.

The FRhK-4 cell line seemed better for virus production than FRhK-6 cultures (Flehmig, 1980). Cytoplasmic immunofluorescence (IF) was shown and the release of 27 nm HAV particles was demonstrated by IEM, but CPE could not be found in HAV-infected FRhK-4 cells (Flehmig, 1980). A persistent infection of HAV, which was stable for at least 12 months, was established in a rapidly growing FRhK-4/R culture and this proved a useful source of antigen for diagnostic tests (Flehmig, 1981).

Hepatitis A virus bands at 1.33 to 1.34 g/ml in a caesium chloride gradient; like poliovirus, it sediments at 160S and is 27 to 32 nm in diameter (Siegl & Frosner, 1978; Coulepis et al, 1982). It was

concluded that HAV should be classified as human enterovirus 72 in the family *Picornaviridae* (Matthews, 1982; Melnick, 1982).

1.3. Picornavirus morphology and morphogenesis

The composition, development and cytopathology of the picornaviruses have been intensively studied. Replication of ribonucleic acid (RNA) is initiated in association with smooth membranes of the cytoplasm and a pool of coat protein is synthesized which exists as "procapsids" ready to envelop newly formed viral RNA (Godman, 1973). The materials of which the progeny virus will be constructed are gathered together in "factory" areas or viroplasm in which assembly and maturation proceed. Self-assembly of capsomeric subunits into procapsids is facilitated by "maturation factors" present in the cytoplasm and viral RNA is rapidly incorporated into virions once sufficient coat protein has been accumulated (Godman, 1973). There may be widespread evidence of autophagy in infected cells.

Granular cytoplasmic masses of viroplasm are associated with crystalline arrays of enteric cytopathic human orphan (ECHO) 9 virus particles. Electron-opaque, electron-translucent and membrane-coated virions, approximately 22 nm in diameter, are seen and virus arrays may be oriented on fibrils (Rifkind et al, 1961). Characteristic particles of ECHO 4 appear in crystalline formation, linear arrays or are randomly distributed (Duffy, Bell & Menefee, 1962). Coxsackie B5 virus particles, 17 to 19 nm in diameter, form chains or cluster in small groups and CPE, the most important feature of which was vacuolization, is evident in all cells (Nunez-Montiel, Vitelli-Flores & Weibel, 1961).

Large crystals of poliovirus types 1 and 2 are seen and virions are

often associated with membranes. The presence of dense cytoplasmic masses serves as a useful indicator in determining whether a particular preparation warrants an exhaustive search for virus particles (Mayor & Jordan, 1962). Infection with poliovirus type 1 induces accumulation of viroplasm and appearance in the matrix of recognizable progeny virions as individuals or in small groups and, later, large paracrystalline arrays. Masses of small membrane-enclosed bodies develop, some of which contain one to several virus particles that are 26 to 28 nm in diameter (Dales et al, 1965).

1.4. Cell line contamination

Several surveys of cell cultures prepared from primate and non-primate tissues have shown that viruses are commonly found as endogenous agents (Malherbe, Harwin & Ulrich, 1963; Hsiung & Atoynatan, 1966; Anderson & Doane, 1972; Swack & Hsiung, 1974). Cultures prepared from monkey tissues yielded the greatest variety of virus isolates and the most frequently used non-human primates for virus studies have been the rhesus (*Macaca mulatta*), cynomolgus (*Macaca fascicularis*) and African green (*Cercopithecus aethiops*) monkeys (Kalter & Heberling, 1968). Continuous cell lines and cultures derived from monkey kidney have been shown to be infected with viruses belonging to the *Polyomavirus* genus of the *Papovaviridae* (for references, please see Table 1.1).

1.5. Papovaviruses

In 1962, Melnick considered that human and rabbit papilloma, mouse polyoma and simian vacuolating viruses shared biological and physical properties and formed a natural group of tumour viruses for which he proposed the name papovavirus. Members of the group cause persistent

infections in the natural host and are oncogenic in experimental animals: several of the viruses from both genera induce a variety of tumours in newborn hamsters and transform cells in culture.

The size of the papovaviruses was thought to be 40 to 50 nm (Melnick, 1962) but Crawford and Crawford (1963) clearly demonstrated that polyoma virus could be differentiated from the papillomaviruses by its smaller size. Because of this and the larger genome of the papillomaviruses subdivision of the group was suggested and the form papovavirus retained as the family name. Polyomaviruses are known to occur in various animal species many of which may be latently infected by more than one antigenic type. The original isolation and morphological descriptions of the polyomaviruses are summarized in Table 1.1. Only those most relevant to the present study will be considered in more detail.

1.6. Simian virus 40 - SV40

1.6.1. Vaccine contamination and short term effects in man

Simian virus 40 (SV40, Table 1.1a) was present in all three types of Sabin's live poliovirus vaccine, which were fed to millions of people of all ages, and in numerous respiratory virus seed stocks used in human volunteer inoculation experiments. Antibody against SV40 was not found in pre-vaccination sera, nor in human gamma globulin, suggesting that natural infection in man occurs rarely, if at all (Sweet & Hilleman, 1960 a & b). No harmful short-term effects that might be attributable to SV40 were reported in field trials of live poliovirus vaccine (Field trials, 1960) and antibody was not detected in children given Sabin's original live attenuated vaccines containing SV40 (Sweet & Hilleman,

Table 1.1a. Original isolation and morphological descriptions of the polyomaviruses

Year	Virus	M/I	Source		Reference
1952	K virus	I	mouse	pooled spleen, liver etc	Kilham, 1952
		M		lung	Dalton, Moore & Mottram, 1959
1953	polyoma	I	mouse	pooled tissue	Stewart, 1953; Gross, 1953
		M		embryo cell culture	Negroni, Dourmashkin & Chesterman, 1959
1960	SV40	I	rhesus & cynomolgus monkey	kidney cell culture	Sweet & Hilleman, 1960 a & b
		M	monkey & human	kidney cell culture	Gaylord & Hsiung, 1961
1963	SA12	I	African green monkey *	kidney cell culture	Malherbe, Harwin & Ulrich, 1963
		M		kidney cell culture	Malherbe & Strickland-Cholmley, 1973
1964	RKV	I	rabbit	papilloma	Hartley & Rowe, 1964
		M		kidney cell culture	Chambers, Hsia & Ito, 1966
1965	PML	M	human	brain	ZuRhein & Chou, 1965
	JCV	M+I		brain	Padgett et al, 1971
	SV40-PML	M+I		brain	Weiner et al, 1972; Penney et al, 1972
1965	FPV	M	frog	kidney	Lunger, Darlington & Granoff, 1965
		M+I		kidney & embryo cell culture	Granoff, Gravell & Darlington, 1969
1968	HPV	M+I	hamster	skin lesion	Graffi et al, 1968
	LHV	M+I		pooled kidney & spleen	Hannoun, Guillon & Chatelain, 1974
1971	BKV	M+I	human	urine	Gardner et al, 1971
1972	SPV	M+I	pig	kidney cell culture/trypsin	Newman & Smith, 1972

continued ...

For table abbreviations, please see 1.1b

Table 1.1b. Original isolation and morphological descriptions of the polyomaviruses

Year	Virus	M/I	Source		Reference
1972	OMKV	M+I	owl monkey	kidney cell culture	King et al, 1972
1974	STMV	M+I	stump-tailed macaque	kidney cell culture	Rangan et al, 1974
1975	M-PML	M	macaque	brain, PML-like lesions	Gribble et al, 1975
1977	HD	M+I	African green monkey	kidney cell culture	Waldeck & Sauer, 1977
1979	LPV	M+I	African green monkey	lymphoblastoid cell culture	zur Hausen & Gissmann, 1979
		M+I	man	lymphoblastoid cell culture	zur Hausen & Gissmann, 1979
1980	WRSV	M+I	calf	kidney cell culture	Coackley, Maker & Smith, 1980
1981	BFDV	M	budgerigar	kidney	Davis et al, 1981
		M+I		skin, liver, kidney etc	Bernier, Morin & Marsolais, 1981; Bozeman et al, 1981
1983	FRKV	M+I	fetal rhesus monkey	kidney cell culture	Richmond, Parry & Gardner, 1983; 1984
1984	CMKV	M+I	cynomolgus macaque	kidney cell culture	Wognum et al, 1984
1984	RPV	M	rat	parotid gland	Ward et al, 1984
1987	CKV	M+I	calf	kidney cell culture	Westcott et al, 1987
1989	PP-2	M+I	baboon	kidney cell culture	Gardner et al, 1989

Year = year the virus was first described. M/I = morphology and/or isolation; M = morphology; I = isolation; M+I = both morphology and isolation

* the principal host is the chacma baboon (Valis et al, 1977)

It is not known if the two polyomaviruses isolated from hamsters, HPV and LHV, are related

1960 a & b; Goffe, Hale & Gardner, 1961; Magrath, Russell & Tobin, 1961).

However, production of SV40 antibody and excretion of the virus have been demonstrated in some recipients of vaccines. Antibody to SV40 was detected in people given formalin-killed adenovirus vaccine (Sweet & Hilleman, 1960 a & b) and in children injected with inactivated (Salk) poliomyelitis vaccine (Sweet & Hilleman, 1960 a & b; Goffe et al, 1961; Magrath et al, 1961). In the Houston trial, children aged three to six months were found to excrete SV40 21 or 28 days after administration of contaminated oral poliovirus vaccine and in the New Orleans trial of the vaccine, infants excreted SV40 between days six and 35 (Melnick & Stinebaugh, 1962).

SV40 was also present in a pool of respiratory syncytial (RS) virus which was used in a study of experimental infection in man. Eight volunteers inoculated by the respiratory route with SV40, in which RS virus had been neutralized, remained free from symptoms in the month following inoculation but developed SV40 neutralizing antibodies. Virus was recovered from throat swabs from three of the volunteers on days seven or 11 indicating subclinical infection (Morris et al, 1961).

Inactivated HAV vaccine used to inoculate 150 newborn infants contained live SV40, 1/10,000 particles of which were resistant to formalin treatment (Hilleman et al, 1981; Robbins 1981). Viable SV40 was also detected in formalinized poliomyelitis and adenovirus vaccines and produced characteristic CPE in all subculture tubes (Gerber, Hottle & Grubbs, 1961).

1.6.2. Possible long term effects of SV40 in man

Shah and Nathanson (1976) suggested that the limited studies undertaken after exposure of the population to infectious SV40 were not sufficient to exclude completely the induction of neoplasms, chronic neurologic disease or other complications arising at a low frequency or after a long interval. SV40 antibodies were found in about 20% of sera from Maryland children born between 1955 and 1957 who had a high risk of having received contaminated Salk poliomyelitis vaccine (Shah et al, 1972). Significant levels of antibody to SV40 remained constant in some subjects for at least three years suggesting the continued presence of antigenic stimulus (Gerber, 1967) and there is conflicting evidence for the long term effects of SV40.

A cohort analysis of cancer mortality data for children who were six to eight years old in 1955, classified according to three dose categories of SV40, revealed no difference in trends (Fraumeni, Ederer & Miller, 1963) but SV40 and SV40 T (tumour) antigens have been detected in the malignant melanoma metastases of a patient in which specific viral and T antibodies were demonstrated (Soriano, Shelburne & Gokcen, 1974). SV40 T antigen was also detected in two meningiomas tested in cell cultures, although it was not shown that the virus was the causative agent (Weiss et al, 1975).

A follow-up study over 17 to 19 years has revealed no deaths from cancer in 1,073 children who received SV40-contaminated poliovirus vaccine compared with the expected rate of one on the basis of the rate in the general population (Mortimer et al, 1981) and Rosa, Sever and Madden (1988) concluded that the association between the administration of killed-poliovirus vaccine to mothers and neurologic tumours in their

offspring was not due to SV40. However, Geissler (1990) found that some intracranial tumours might be more frequent among persons who have been given contaminated vaccine and recommended that epidemiological evaluation should continue for at least two more decades before definitive conclusions could be drawn about the possible involvement of SV40.

1.7. Stump-tailed macaque kidney virus - STMV

In 1974 Rangan and co-workers described the isolation of STMV (Table 1.1b) from kidney cultures of 15 stump-tailed macaques (*Macaca arctoides*). All cultures developed cytoplasmic vacuolation; often this was transient, occurring after the cells had been passaged a few times. The CPE, which resembled that of foamy virus or SV40, could not be passed to several other cell lines (Rangan et al, 1974).

Subsequently STMV was identified as a new polyomavirus by Reissig and her colleagues (1976) and was grown in rhesus monkey kidney cells. Using IEM and IF it was demonstrated that STMV was distinct from polyoma virus, SV40, JCV and BKV although its tumour antigen was related to that of SV40 and BKV. The virions were irregularly and incompletely coated by a fine amorphous material (Reissig et al, 1976).

STMV was thought to be unique amongst the polyomaviruses in that the virus was always present in kidney cultures from stump-tailed macaques of all ages (Shah et al, 1977). The high frequency of isolation of STMV was not thought to be due to laboratory cross-contamination since some of the kidney biopsies had been independently maintained (Rangan et al, 1974). It was suggested that STMV is normally congenitally transmitted (Shah et al, 1977) and, since antibody was not present in animals from

which virus was isolated, that the virus remains unexpressed (Shah et al, 1975).

1.8. HD polyomavirus - isolated in Vero culture

In 1977 Waldeck and Sauer showed that a Vero cell line, derived from *Cercopithecus aethiops* (African green monkey) kidney, was chronically infected with polyomavirus HD (Table 1.1b). Vero cells which harboured HD virus did not display any CPE and HD virus deoxyribonucleic acid (DNA) was not detected in Vero cells from other sources. The virus grew in LLC cells of rhesus monkey origin, also without producing a CPE, but did not grow in bovine cells (Bosslet & Sauer, 1978).

HD virus, which is capable of rapidly transforming Vero cells *in vitro*, was shown by DNA-DNA hybridization studies to be unrelated to polyoma virus, SV40, JCV and BKV (Waldeck & Sauer, 1977). The virus was shown to be indistinguishable from STMV by IF reactivity, restriction endonuclease analysis and nucleic acid hybridization assay (Howley et al, 1979).

1.9. Wokalup Research Station virus - WRSV

The presence of polyomavirus WRSV (Table 1.1b) in a stock of primary kidney cells from a healthy newborn colostrum-deprived calf, stored in liquid nitrogen, was demonstrated on several occasions. The virus had no effect on cell multiplication and at no time was any CPE observed. The presence of eosinophilic intranuclear inclusion bodies in about 10% of cells and the detection of polyomavirions by EM were the only indications of virus multiplication (Coackley et al, 1980). WRSV was readily passaged in calf kidney (CK) cells without CPE but failed to

grow in pig kidney, calf testis, monkey kidney (Vero), hamster kidney (BHK-21) and feline kidney. The virus did not agglutinate red cells from chickens, sheep, guinea pigs or humans (Coackley et al, 1980).

1.10. Cynomolgus macaque kidney virus - CMKV

Negative stain revealed the presence of a polyomavirus, CMKV, in uninoculated cynomolgus macaque kidney cells (Table 1.1b). Extensive CPE was evident after six to eight weeks in culture (Wognum et al, 1984). Without inducing CPE the virus also replicated in BSC-1, Vero, human embryonic and calf kidney cells. Based on genome size and restriction endonuclease data, the virus was identifed as a new isolate of STMV (Wognum et al, 1984).

1.11. Calf kidney virus - CKV

Polyomavirus, CKV, was isolated from the kidneys of 18 of 64 clinically normal calves from seven different farms (Westcott et al, 1987). Kidney cultures were grown using gamma-irradiated fetal calf serum, lamb serum or horse serum. Initially the virus took about six weeks to produce a CPE, which consisted of vacuolation. Eosinophilic intranuclear inclusions were seen and negative stain revealed polyomavirions (Westcott et al, 1987).

1.12. Identification of fetal rhesus kidney virus - FRKV

In some negatively stained preparations of culture fluid antibody can be seen attached to the polyomavirions by EM. The amount of antibody is determined by the concentration of fetal or newborn calf serum in the medium and the number of virions observed. When serum-free medium is

used, antibody is not observed.

Using IEM I shall show that FRKV is not SV40, SA12, JCV or BKV, but is indistinguishable from STMV and hence HD. The virus is also indistinguishable from CMKV and is antigenically similar to the LLC-MK2 polyomavirus. I shall demonstrate that the virus reacts in IEM with antibody in fetal, newborn and adult bovine sera. FRKV is identical to WRSV and is closely related to two of the CK isolates. I shall show that a batch of fetal bovine serum contains both antibody and infectious virus (or possibly infectious DNA). Since other batches of fetal and newborn sera contain antibody, these might also be infectious.

I shall seek to prove that FRKV, STMV and hence HD, CMKV and LLC-MK2V are several isolates of the same bovine virus, WRSV, and that these monkey cell lines have become infected through the use of contaminated bovine serum.

This thesis will demonstrate the importance of EM in the discovery and identification of the virus. The growth of HAV in FRhK-4 cells was being monitored by light microscopy, IF and radioimmunoassay (RIA), thus without electron microscopy FRKV was unlikely to have been discovered. Furthermore, since the presence of bovine antibody attached to virions was the only indication of the source of the polyomavirus, the origin of FRKV, together with that of STMV, HD and CMKV, was unlikely to have been determined without electron microscopy.

2. MATERIALS AND METHODS

2.1. Cell culture and propagation of viruses

FRhK-4 cultures, pass 76, obtained from B Flehmig (Tubingen University, Germany), were grown at 37°C on Eagle's minimum essential medium (MEM) containing 6% or 10% fetal bovine serum (FBS) (Flow Laboratories) and maintained at 33°C or 37°C on MEM with 2% FBS which was changed at approximately weekly intervals (J V Parry). FRhK-4 (passes 21 and 42), FRhK-6 (pass 5), DBS-FRhL-2 101 and DBS-FRhL-2 103 cells, supplied by J C Petricciani (Bureau of Biologics, Bethesda, USA), were grown and maintained in separate laboratories (J V Parry, S D Gardner and P E Gibson) from FRhK-4 cells, with their own media, to reduce the risk of contamination with the FRhK-4-derived polyomavirus.

FRhK-4 cells were passaged twice at approximately monthly intervals before inoculation with HAV (J V Parry) and the production of hepatitis A antigen (HAAg) was monitored by solid phase radioimmunoassay (RIA). Maximum HAAg was detected (Parry, 1983) after 42 days of incubation at 37°C at which time culture fluids from both HAV-inoculated and control uninoculated cells were examined by EM and found to contain polyomavirions.

To further investigate this polyomavirus, an uninoculated stock culture of FRhK-4 cells was maintained at 33°C for 6 to 8 weeks before subdivision when several extra cultures were made. FRhK-4 and FRhK-6 cells were maintained at 37°C on either MEM with 2% FBS or Iscove's modification of Dulbecco's MEM without serum (J V Parry). Some cultures were maintained on serum-free medium (Gibco) (P E Gibson) and some on medium containing equine serum (S D Gardner). The culture fluids, cells which spontaneously detached from the cell sheet and monolayer cells were used as sources of polyomavirus for all investigations.

2.2. Viruses and sera

Human HAV for growth in FRhK-4 cultures was supplied by F Burkhardt (Bern University, Switzerland). The MS-1 strain of human HAV, provided by A J Zuckerman (School of Hygiene and Tropical Medicine, London) was passaged in marmosets by H Appleton who also prepared the 10% faecal extract in phosphate buffered saline (PBS). WRSV was supplied by W Coakley (Animal Health Laboratory, South Perth, Western Australia) and grown in secondary CK8 or CK26 cells by S D Gardner; in cultures for IEM, maintenance medium contained 2% equine serum instead of bovine serum. Polyoma virus and antiserum were provided by L V Crawford (Imperial Cancer Research Fund, London) and the LLC-MK2 culture fluid by B M Totterdell (St Thomas's Hospital, London).

Two STMV antisera, 75/176 and 81/239, prepared in rabbits and one in a rhesus monkey were provided by K V Shah (Johns Hopkins University, Baltimore, USA) who also supplied rabbit antisera against SA12 and SV40. J Hill (Pfizer Ltd, Kent) provided the calf SV40 antiserum and C J A Sol (University of Amsterdam, The Netherlands) the monkey antiserum against CMKV. Guinea pig antisera against the human polyomaviruses BKV and JCV, and three rabbit antisera against FRKV were raised in the Virus Reference Division by S D Gardner. Purified preparations of goat and rabbit anti-bovine immunoglobulin G (IgG) (Sigma) were also used.

Several bovine serum pools supplied for cell culture (Flow Laboratories or Gibco) were examined. These included fetal bovine sera 11/3, 24/8, 18/3 and 14/10; newborn bovine sera 17/8, 22/6 and 21/6; and adult bovine serum 30/3. In addition, various sera from individual animals were used.

2.3. Electron microscopy (EM) negative stain

Negatively stained virus particles were first illustrated by Hall (1955) and Huxley (1957) who observed that, if excess stain was not completely removed by washing, virions became surrounded and outlined by the dried reagent. Both Hall and Huxley commented on the usefulness of the images thus obtained, but it was not until 1959 that Brenner and Horne described the "simple" method of "embedding" virus particles in an electron dense material which introduced contrast by negative staining. It is almost unbelievable that the description of the technique which has contributed so much to the understanding of virus morphology was rejected for publication by the journal Virology because it "was unlikely to reveal any additional features of viruses which could not be determined by existing preparative procedures" (Horne & Wildy, 1979).

High contrast with good preservation was obtained by mixing virus preparations with 1% phosphotungstic acid (PTA) adjusted to pH 7.4 with potassium hydroxide (Brenner & Horne, 1959) and this method, with minor modifications, has been used in the present study.

For the examination of cells that had become spontaneously detached from the monolayers during incubation, culture fluids (5 mls) were centrifuged at 1,500 g for 10 minutes. Monolayer cells were washed with phosphate buffered saline (PBS), scraped off the flasks into PBS then similarly centrifuged. High speed pellets were prepared by centrifuging 3 or 5 ml volumes of culture fluids or clarified culture fluids at 48,000 g for 1 hour or at 150,000 g for 2 hours. All pellets were resuspended in small volumes of distilled water (approximately 20 ul), applied to Formvar/carbon-coated grids and negatively stained with 1-3% PTA, pH 6.3.

For extraction of virus, detached cells from 5 to 25 ml volumes of culture fluids were centrifuged at 1,500 g for 10 minutes followed by resuspension in PBS then recentrifugation. The pellets were resuspended in 20 to 40 ul distilled water, treated in an ultrasonic bath for 2 minutes, recentrifuged, and the supernatants negatively stained.

Attempts to purify the virus extracted from cells by treatment with Nonidet P40 (Newman & Smith, 1972) for 30 minutes or by homogenization with fluorocarbon (Howatson & Almeida, 1960b; Mattern, Allison & Rowe, 1963; Black, Crawford & Crawford, 1964; Schwerdt et al, 1966; Reissig et al, 1976; Muller & Nitschke, 1986) followed by centrifugation were also made.

2.4. EM thin sections

2.4.1. Fixation

Monolayers of HAV-inoculated or control FRhK-4 cells were fixed *in situ* with 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.4 (Sabatini, Bensch & Barrnett, 1963) for 1-2 hours at 4°C. The cells were scraped off the plastic with a spatula covered with Nescofilm into fresh fixative. Suspensions of spontaneously detached cells were similarly fixed with cacodylate-buffered glutaraldehyde. Samples were centrifuged in an Eppendorf centrifuge at 15,000 g for 2 minutes, the resulting pellets washed 3 times in 0.25 M sucrose - 0.1 M cacodylate solution (Sabatini et al, 1963), cut into fragments and maintained overnight at 4°C. The blocks were postfixed in cacodylate-buffered (Trump & Ericsson, 1965) 1% osmium tetroxide for 1 hour on ice then briefly washed with distilled water before dehydration.

2.4.2. Dehydration and embedding

Fragments of pellets were dehydrated in a graded series of aqueous ethanol solutions (50%, 75%, 90%) then in 3 changes of absolute ethanol followed by 2 changes of propylene oxide. Epikote 812 epoxy resin was mixed with accelerator in the proportion of A:B 4:6 according to Luft (1961), centrifuged at 1,500 g for 10 minutes to remove air bubbles and either used immediately or after storage at -30°C (Minick, 1963). Specimens were rotated during infiltration in a 1:1 mixture of propylene oxide and epoxy resin for 1 hour at room temperature (RT) then 2 hours at 37°C, followed by 1 hour at 37°C in resin alone. Blocks were drained on absorbent tissue paper before being transferred to dried, size 0 gelatin capsules containing fresh epoxy resin which was polymerized at 60°C for 4-6 days.

2.4.3. Sectioning and staining

A Reichert OMU2 ultramicrotome was used. Ultrathin sections with gray to silver interference colours, 60-90 nm (Peachey, 1958), were cut with a diamond knife and mounted, after spreading with chloroform vapour (Sotelo, 1957), on cleaned 400 mesh copper grids which had been soaked briefly in a very dilute solution of Sellotape adhesive (Pease, 1964). Thin sections were contrast stained for 10 minutes in a saturated solution of uranyl acetate in 50% ethanol (Watson, 1958) followed by 2 minutes in lead citrate (Reynolds, 1963) and washed thoroughly in several changes of distilled water.

2.5. Electron microscopy and photography

Specimens were examined in a JEOL 100CX or Philips 420T electron microscope using the magnifications of x 66,000 or x 62,500 for

negatively stained preparations and x 50,000 or x 51,000 for thin sections. Electron micrographs were taken on Kodak or Ilford EM cut film using an accelerating voltage of 80 kV and an exposure time of 2 seconds with an appropriate level of illumination determined by the photometer. Negatives were developed in Kodak D19 developer for 2 minutes at 20°C and photographic enlargements made using a Leitz II C or De Vere 504 enlarger with tungsten bulbs. An Ilfospeed 4250 autoprocessor with resin-coated paper and Ilfospeed solutions were used. With few exceptions, micrographs of negatively stained preparations have been printed at a final magnification of x 200,000 and those of thin sections at x 30,000 or x 150,000.

In attempting to optimize EM performance (Chapman, 1980), consistent procedures for microscopy were adopted, most of which are outlined below (Section 2.6). The two factors which most influenced the quality of the micrographs were correction of objective lens astigmatism close to the area being photographed and slight underfocussing of the image. The practice which contributed most to improving quality was the critical examination of all negatives on a light box with a magnifier.

2.6. Magnification calibration and measurement of FRK4V and papillomavirus particles

The microscope magnification was calibrated using glutaraldehyde-fixed beef liver catalase which had been recrystallized and provided by N G Wrigley (National Institute for Medical Research, Mill Hill) (Wrigley, 1968). Human papillomavirus was partially purified from plantar wart by P E Gibson. Suspensions of catalase, FRhK-4-derived polyomavirus and human papillomavirus were negatively stained with PTA

as described above.

The microscope (JEOL 100CX) was aligned and liquid nitrogen was used to minimize contamination. Grids were loaded in the holder with the specimen away from the electron beam and suitable catalase crystals or virus particles near the centre of the grids were carefully selected. The height of each specimen was adjusted to the eucentric position to set the objective focal length to a constant value and standardize its magnification factor.

Before every exposure, the objective lens astigmatism was corrected on the background grain at maximum magnification (x 250,000) and, to minimize the effects of hysteresis, the selected magnification of x 66,000 was always approached from x 250,000. To improve electron beam coherence the illumination was adjusted by condensing the beam from overfocus. At the point of focus, the change in the image which occurs when overfocussed Fresnel fringes disappear is easier to detect than that which results from the disappearance of underfocussed fringes. The slight amount of underfocus desired was therefore always approached from overfocus.

As recommended by Wrigley (1968), the inclusion of a catalase crystal in each field of virions would, of course, provide the ideal internal standard of measurement. However, such an approach is impractical: it requires highly concentrated virus suspensions, an unlimited supply of purified catalase and, because the illumination necessary to show the crystal lattice clearly differs significantly from that required by the virus, 2 exposures of every field! A more practical method was therefore devised: micrographs of 12 different catalase crystals were taken, 6 before and 6 after those of the virus preparations, and all

micrographs used for measurement were taken in one session.

Shrinkage of resin-coated paper is minimal and care was taken to prevent errors due to incorrect enlarger settings. Photographic enlargements were made in one session at the beginning and end of which the enlarger magnification was checked by printing a scale. As many lattice lines as possible, usually about 100 per catalase crystal, and the diameters of 100 morphologically characteristic virions from each virus preparation were measured on the prints. A measuring magnifier with a graticule was used and, since virus particles were rarely round, each virion was measured along, and perpendicular to, its longest dimension. The print was then marked to prevent repeated measurement of the same virion. Damaged and aberrant forms were not included and almost all of the particles measured appeared "full" (Crawford & Crawford, 1963).

The principal lattice spacing of catalase determined by low-angle electron diffraction (Wrigley, 1968) is 17.5 nm and the 8.75 nm half-spacing, or distance between lattice lines, was used to calculate the true microscope magnification.

2.7. Virus isolation and haemagglutination

Culture fluid from FRhK-4 cells (pass 78) incubated for 42 days was inoculated into primary human embryo kidney (HEK) cells (S D Gardner). The cultures were maintained on Eagle's MEM with 2% newborn bovine serum (NBS) and incubated at 39^{o}C for 1 month. The medium was changed three times a week. Subcultures were made from the inoculated HEK cultures, which were showing cytopathic changes, either directly from the medium or from infected cells which had been frozen and thawed three times then ultrasonicated to release virus particles. FRK4V and HEK-adapted FRK4V

were also inoculated into secondary cultures of calf kidney (CK) cells.

Suspensions of FRK4V for haemagglutination (S D Gardner) were treated with either Nonidet P40 (BDH Chemicals Ltd) for 30 minutes at 37°C or were incubated overnight with neuraminidase and then inactivated at 56°C for 30 minutes. Treated virus preparations were warmed at 37°C for 30 minutes before testing for the ability to agglutinate 0.5% human O cells at 4°C, pH 6.3 and 0.5% bovine red cells at 4°C and 37°C, pH 6.3. Untreated virus was also tested for haemagglutination with 0.5% suspensions of erythrocytes from chicken, sheep, rhesus monkey, baboon and man at 4°C, 20°C and 37°C, pH 7.0.

2.8. Immunoelectron microscopy (IEM)

The immunological reactions between viruses and their specific antisera were first observed by Anderson and Stanley (1941) and von Ardenne, Friedrich-Freska and Schramm (1941) who demonstrated the potential value of EM in the study of antigen-antibody interactions. Immunoelectron microscopy (IEM) was not widely used, however, until after the introduction of negative staining (Brenner & Horne, 1959) which allowed antibody molecules surrounding and aggregating the virions to be clearly seen (reviewed by Almeida & Waterson, 1969).

The technique has frequently been applied to the study of papovaviruses (Almeida & Waterson, 1969; Gardner et al, 1971; Penney et al, 1972; Albert & ZuRhein, 1974; Field et al, 1974; Reissig et al, 1976) and usually involves incubating 0.1 ml volumes of virus mixed with antiserum for 1 hour at RT or 37°C followed by dilution with PBS and maintenance overnight at 4°C before the immune complexes are pelleted by high speed centrifugation. To reduce the volumes of virus required and eliminate

centrifugation, a micro IEM method was developed. Equal volumes (2.5ul) of antigens and sera were mixed on Nescofilm and maintained in a moist chamber for 1 hour at RT. The mixture was applied to a Formvar/carbon-coated grid then negatively stained with 1-3% PTA. In general, the terms used to describe IEM reactions (antibody coating and clumping) are those suggested by Roberts, Milne and van Regenmortel (1982).

Human HAV grown in FRhK-4 cells was compared in IEM tests with that in the faecal extract from marmosets inoculated with the MS-1 strain of HAV. Anti-HAV IgG was purified from a high-titre human serum by fractionation on DE52 ion-exchange gel (J V Parry, 1981).

All preparations of FRK4V for IEM were from uninoculated FRhK cultures: suspensions of virus extracted from spontaneously detached cells, or free virus from fluids from cultures maintained on serum-free medium were used. FRK6V from uninoculated FRhK-6 cultures was used for as many tests as possible, after which FRK6V grown in CK17 cells was used. WRSV was grown in CK cultures, SV40 and SA12 were grown in Vero cells. The appearance of the virus in all preparations was examined by EM before use. Antigens and sera were diluted in PBS. Specific antisera against SV40 and SA12 were used at the dilutions that resulted in dense antibody coating of their homologous viruses. Other antisera were known to react positively in IEM tests (Table 3.1). Most IEM tests were repeated on different occasions with different batches of antigens and the results are summarized in Section 3.17.

2.9. Immunofluorescence (IF)

FRKV antigen for use in IF tests (S D Gardner) was prepared either directly from FRhK-4 cells or from CK cells inoculated with FRK4V. The

cells were grown as monolayers in multiwell chambers and incubated at 37°C in a CO_2 incubator. Between 6 and 19 days after seeding of FRhK-4, or post infection of CK, the coverslip cultures were fixed in acetone at RT for 20 minutes and stored at -30°C until required. FRK4V-infected cells and control uninoculated CK cells were examined with type specific sera in an indirect method using fluorescein-labelled antibody against rabbit and human immunoglobulins (Wellcome Reagents Ltd).

2.10. EM negative stain of counter-immunoelectrophoresis (CIE) precipitin lines

The technique used to examine CIE antigen precipitates was essentially as described by Watson and his co-workers (1966) for polyoma virus in gel diffusion. Precipitin lines were cut from CIE gels with a scalpel and homogenized in 20-40 ul volumes of distilled water. The homogenate was applied to Formvar/carbon-coated grids and negatively stained with PTA.

Immunoglobulin was precipitated by ammonium sulphate from NBS 22/6 (J V Parry) then further purified both by protein A-sepharose affinity chromatography and ion exchange using DE52 (Whatman) under IgG eluting conditions. Three serum samples, including NBS 22/6, were mixed 1:4 with receptor destroying enzyme (RDE) (Wellcome Reagents Ltd) then incubated at 37°C for 1 hour.

In addition to purified bovine IgG and RDE-treated sera, fetal and newborn serum pools, sera from individual cattle of various ages and the rabbit FRKV antisera were tested in CIE with FRK4V. The method used was that of Cohen, Hewish and Mortimer (1981); NBS 22/6, which was positive for anti-FRKV by IEM, was included on every plate.

2.11. Antibody-coated grids (ACG)

In 1973 Derrick described a method by which virus particles were specifically attracted to and concentrated on the surface of antibody-coated grids. For each dilution of tobacco mosaic virus (TMV) tested, 40 to 50 times more virions were attached to antiserum-treated grids than to control grids. The technique has also been used for several animal viruses (Almeida, Stannard & Shersby, 1980; Giraldo et al, 1982; Pegg-Feige & Doane, 1983; Kjeldsberg & Siebke, 1985; Venuti et al, 1985). The method has been adapted in an attempt to specifically attract antibody-coated polyomavirus particles and thereby increase the sensitivity of EM. Formvar/carbon-coated grids were floated on various dilutions of goat (GAB) or rabbit (RAB) anti-bovine IgG in PBS for 30 minutes at RT, washed with PBS or distilled water then drained. Antibody-coated grids were then floated on 3 ul drops of a preparation of culture fluid, containing very small numbers of densely antibody-coated polyomavirions, for 1 hour at RT, drained then negatively stained with PTA.

2.12. Long term incubation of calf kidney, CK, cultures

Cultures were prepared each week from calf kidneys by J R Lukey (Central Veterinary Laboratory, Weybridge) who used gamma-irradiated FBS in the media to prevent contamination with bovine diarrhoea virus (BDV). Thus on receipt, CK cultures had not been in contact with unirradiated FBS. Cells were subcultured on arrival using 10% FBS in growth medium then 2% FBS in maintenance medium (J V Parry and S D Gardner). Culture fluids were harvested weekly and stored at $4^{o}C$; as many harvests as possible were examined by negative stain EM for the presence of polyomaviruses.

Fetal bovine serum U720201D, which had not been gamma-irradiated, was used in all media for the first 19 batches of cells received, CK8 to CK28. CK29 cells were subcultured using 10% FBS U720210D then divided into 2 batches: maintenance medium for CK29/1 contained gamma-irradiated FBS whereas that for CK29/2 cells contained equine serum. Gamma-irradiated FBS was used throughout for CK30 cultures.

CK31, CK32, CK33 cells were subcultured using 10% gamma-irradiated FBS then each divided into 2 batches. CK31/1, CK32/1 and CK33/1 were exposed to 2% FBS U720201D for 4, 2 and 6 weeks respectively after which the suspect serum was replaced with 2% gamma-irradiated FBS. Maintenance media for CK31/2, CK32/2 and CK33/2, and all media for CK34 to CK42 contained gamma-irradiated FBS.

An RIA test for FRKV antigen was subsequently developed (Parry & Gardner, 1986) and the last harvest from each CK culture was later tested by this method.

3. RESULTS

3.1. HAV: morphology and IEM with purified anti-HAV IgG, Figures 1 to 3

Only rare, scattered picornavirus particles, approximately 27 nm in diameter, could be found in preparations from HAV-inoculated FRhK-4 cultures after prolonged incubation (Fig 1), despite the large amount of hepatitis A antigen detected by RIA. Cytopathic effects (CPE) were not observed but a few cells became detached from the monolayers. Similar numbers of picornavirions were seen in low speed pellets of such spontaneously detached cells and in high speed pellets of the resulting clarified culture fluids from HAV-inoculated cells. Varying the time interval between subculture and harvesting from 37 to 128 days did not affect the numbers of virus particles detected.

Picornavirions could not be found in control, uninoculated FRhK-4 cells either in low speed pellets of culture fluids or in high speed pellets of clarified culture fluids.

When preparations of low speed pellets of culture fluids from HAV-infected FRhK-4 cells were incubated with purified anti-HAV IgG, small numbers of picornavirus particles with dense antibody coating were seen (Fig 2). Not only were greater numbers of picornavirus particles observed in IEM preparations but single scattered virions were easier to detect. Such antibody-coated picornavirions, which also occurred in small clumps (Fig 2), were morphologically indistinguishable from those observed when faecal extracts from marmosets infected with the MS-1 strain of HAV were mixed with the same IgG preparation (Fig 3).

"Clean" picornavirus particles, like those in Figure 1, could not be found after either antigen had been incubated with anti-HAV IgG.

3.2. Detection of a polyomavirus in control FRhK-4 cultures, Figures 4 to 6

During the search for hepatitis A particles, polyomavirions were unexpectedly detected in spontaneously detached cells from FRhK-4 cultures (pass 78) after 42 days of incubation (Fig 4). Such polyomavirus particles, approximately 47 nm in diameter, were present in similar numbers in both HAV-inoculated and control, uninoculated FRhK-4 cultures and were also found in culture fluids from uninoculated FRhK-4 cells that had been maintained in the Virus Laboratory at the Middlesex Hospital (Figs 5 & 6).

Polyomavirions were detected after seven to 155 days of incubation, larger numbers of particles being observed when cultures were maintained for longer periods without subdivision (Figs 5 & 6). However, polyomavirus particles could not be found in culture fluids from FRhK-4 cells that were repeatedly subdivided at 21 day intervals, nor from any other cell lines concurrently maintained in the Virus Reference Division.

3.3. Effect of method of preparation on the appearance of polyomavirus particles, Figures 4 to 8

Moderate to large numbers of polyomaviruses were seen in low speed pellets of cells that had become spontaneously detached from the monolayers during culture (Figs 4-6), but virus particles were rarely found in negatively stained preparations of monolayer FRhK-4 cells which remained attached to the substrate. In all preparations, FRhK-derived virus particles occurred singly and in clumps (Figs 4-6) and were sometimes attached to fragments of membrane (Fig 5). There were

occasional polyomavirus filaments, small numbers of damaged particles, and "mini" spherical polyomavirus particles approximately 38 nm in diameter (Fig 6). Small, round or oval, featureless particles of various sizes (Fig 6) were also observed. In addition, fine strands (Fig 4) or amorphous substance (Fig 5) that did not resemble antibody, often seen between the polyomavirus particles, sometimes appeared to be holding the virions together in aggregates (Figs 4 & 5).

High speed pellets of culture fluids that had not been clarified, comprising virus in spontaneously detached cells and free virus, contained mixtures of "clean" polyomaviruses, like those illustrated in Figures 4 to 6, and polyomavirions coated with antibody (Figs 7 & 8). Small numbers of virus particles, almost all of which were antibody-coated, were observed in high speed pellets of clarified culture fluids.

The antibody surrounding polyomaviruses (Figs 7 & 8) was morphologically indistinguishable from that coating picornavirions after HAV antigen had been incubated with purified anti-HAV IgG (Figs 2 & 3). Antibody-coated polyomaviruses could not be found in low speed pellets of culture fluids.

After subdivision, FRhK-4 cells were cultured on growth medium containing 10% fetal bovine serum for seven days at which time densely antibody-coated polyomavirions (Fig 7) were seen in high speed pellets of culture fluid. When similarly prepared culture fluid from the same bottle of cells was harvested at day 25, the cells having been maintained on medium that contained 2% fetal bovine serum for 18 days, only small to moderate amounts of antibody could be found surrounding virus particles (Fig 8).

3.4. Detection of a polyomavirus in uninoculated FRhK-6 cultures, Figures 9 to 12

Virus particles were not detected in the FRhK-6 cells (pass 5) originally obtained from the USA when examined after eight weeks in culture. However three months later, during which time the cells had been subdivided twice, moderate to large numbers of polyomavirions were seen after incubation for 21 days (Figs 9-12). In some harvests, only very small numbers of polyomavirions could be found.

"Clean" virus particles were found in preparations of detached cells (Figs 9-11), and particles with antibody attached were found in high speed pellets of clarified culture fluids (Fig 12). Damaged polyomaviruses and amorphous substance (Fig 10), "mini" polyomavirions (Figs 10 & 12), polyomavirus filaments (Figs 9, 11 & 12), virions associated with fragments of membrane (Fig 9) and small roundish particles (Figs 10-12) were all observed.

3.5. Detection of polyomaviruses in two uninoculated low pass FRhK-4 cultures and DBS-FRhL-2 101 cell line, Figures 13 to 15

Virus particles could not be found initially in either of the low pass FRhK-4 cultures obtained from the USA. However, after five months incubation polyomavirions were seen in pass 25 (Fig 13) and eight antibody-coated polyomavirus particles were found in pass 44 after subculture for 21 days (Fig 14). Small round particles (Fig 13) and polyomavirus filaments were seen; variable amounts of antibody (Fig 14) were attached to the virus in high speed pellets of clarified culture fluids.

The two fetal rhesus lung cell lines DBS-FRhL-2 101 and DBS-FRhL-2 103, also obtained from the USA, were maintained for more than 54 weeks during which time high speed pellets of culture fluids were examined at approximately fortnightly intervals. On one occasion, a single antibody-coated polyomavirion was detected in an uninoculated DBS-FRhL-2 101 culture after 21 weeks incubation. At no time could polyomavirus be detected in high speed pellets of culture fluids from uninoculated DBS-FRhL-2 103, but 28 days after inoculation of this cell line with fluid from FRhK-4 culture a few polyomavirions were found (Fig 15).

3.6. FRK4V passaged in human embryo kidney (HEK) cells and antibody coating with newborn bovine serum 17/8, Figures 16 to 21

Cytopathic effects were first evident in HEK cells 21 days after inoculation with FRhK-4-derived virus (FRK4V) and consisted of early cytoplasmic vacuolation followed by granular rounding of cells which detached from the cell sheet (S D Gardner). Highly refractive cells were also observed. On passage the interval before appearance of the CPE was reduced to nine days.

Haemagglutination was not observed when FRK4V was mixed with human O, chicken, bovine, sheep, rhesus monkey or baboon erythrocytes (S D Gardner).

Moderate to large numbers of polyomavirus particles were detected in high speed pellets of HEK culture fluids 16 days after inoculation with FRK4V (Fig 16) and at various time intervals thereafter (Figs 17-19). Virus particles appeared either "clean", associated with amorphous substance that did not resemble antibody (Fig 16), attached to or occasionally completely surrounded by membrane (Fig 17) or densely

coated with antibody (Figs 18 & 19). Preparations of monolayer cells from FRK4V-inoculated HEK cultures contained very small numbers of polyomavirus particles that were "clean" (Fig 20) or membrane-associated (Fig 21). Evidence of polyomavirus could not be found in uninoculated HEK cultures however these were prepared.

3.7. FRK4V passaged in calf kidney cells, CK8, Figure 22

Both FRK4V obtained directly from FRhK-4 cells and HEK-adapted virus grew readily when inoculated into secondary calf kidney CK8 cells, with an incubation period of nine to ten days. Early cytoplasmic vacuolation, which was extensive in these cells, was followed by granular degeneration (S D Gardner). Large numbers of spherical polyomavirions, filamentous particles, amorphous substance and small round particles were present (Fig 22). Virus particles were not observed in high speed pellets of control CK8 culture fluids at days nine or ten.

3.8. FRK6V passaged in CK17 cells and CK17 virus, Figures 23 and 24

Polyomavirus FRK6V, obtained directly from FRhK-6 culture, also grew readily in secondary CK cells. Large numbers of "clean" polyomavirions were present in detached cells and high speed pellets of clarified culture fluids from CK17 cells 39 days after inoculation with FRK6V. Despite extensive searching, virus particles could not be found in such preparations of the control, uninoculated CK17 culture at day 39.

After a further 20 days incubation, when very large numbers of polyomavirions were present in FRK6V-inoculated CK17 culture (Fig 23), polyomavirus particles were detected in both detached cells (Fig 24) and

high speed pellets of clarified culture fluids from the uninoculated CK17 control.

3.9. FRhK-4 cells on Iscove's serum-free medium, Figures 25 and 26

Because of the amorphous substance and the variable amounts of antibody attached to some of the polyomavirions harvested from FRhK-4 and FRhK-6 culture fluids, attempts were made to "launder" the virus. However, treatment of such samples with Nonidet P40 or fluorocarbon depleted the numbers of virus particles without improving their appearance. Thus the use of such substances for purification of antigen for IEM was considered impractical. Serum-free medium was used for some low pass FRhK-4 cultures, but virus particles were not always "clean" and occurred mainly in clumps. Furthermore, the yield of virus detected by EM was significantly reduced.

Iscove's serum-free medium proved more satisfactory and with it small to moderate numbers of "clean" polyomavirions were obtained from detached cells (Fig 25) and from high speed pellets of clarified culture fluids from FRhK-4 (Fig 26) and FRhK-6 cultures. However, clumps of virus particles occurred in all preparations and sometimes virions seemed to be held together by amorphous substance (Fig 25). Evidence of antibody could not be found in any of the preparations from cultures maintained on Iscove's medium.

3.10. Papillomavirus morphology, Figures 27 to 29

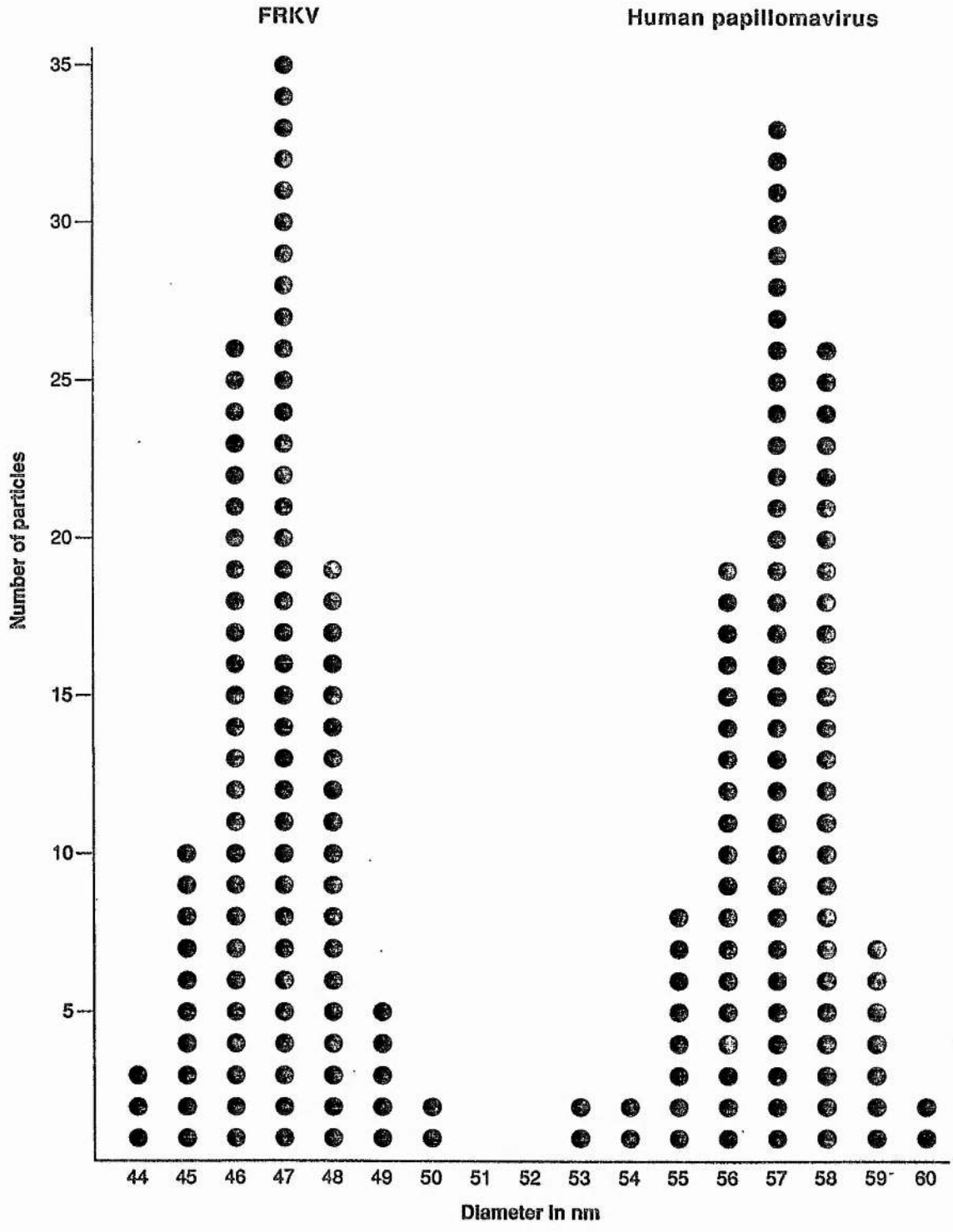
Very large numbers of papillomavirions were present in the partially purified preparation of human plantar wart (Figs 27-29). Most of the virions were "clean" although there was some evidence of fine strands

between some of the particles in aggregates. Antibody was not observed but varying amounts of amorphous substance were seen (Figs 27-29). In addition to "normal" spherical virus particles there were small numbers of papillomavirus filaments, some of which were narrow (Fig 29).

3.11. Measurement of FRK4V and papillomavirus particles

Human papillomavirus and virus from FRhK-4 cultures, passaged in HEK then CK8 cells, were compared. Most of the virus particles observed in these preparations were morphologically characteristic and icosahedral. Papovavirus filaments, "mini" spherical and damaged particles were not included when virions were measured. The range and distribution of the diameters of 100 profiles of each virus are shown in the histogram. The mean diameter of FRK4V was 46.8 nm; that of papillomavirus was 57.1 nm.

Diameter of FRKV compared with that of human papillomavirus



The microscope magnification was calibrated using negatively stained crystalline catalase and the measurements corrected accordingly

3.12. Polyomaviruses in thin sections of FRhK-4 cells, Figures 30 to 34

Polyomavirus particles were observed in 5% to 10% of the FRhK-4 monolayer cells examined after 55 days of incubation. Polyomavirions, approximately 40 nm in diameter, were seen in both HAV-inoculated (Figs 30 & 31) and control, uninoculated (Figs 32-34) FRhK-4 cultures. Approximately equal numbers of morphologically undamaged (Fig 30) and degenerate (Figs 31 & 32) cells contained polyomavirions, which were usually found within the nuclei (Figs 30-32).

In most polyomavirus-containing cells examined small numbers of virions were scattered throughout the nucleoplasm whereas larger numbers of virus particles were randomly arranged in loose groups (Fig 30). A single paracrystalline array of polyomavirions was observed within the nucleus of a degenerate cell (Fig 32) and polyomavirus filaments were occasionally seen (Fig 33).

In addition to intranuclear virus, a few cells contained polyomavirus particles that were either free within the cytoplasm or closely associated with cytoplasmic membranes (Fig 34).

3.13. Picornavirus-like particles in thin sections of HAV-inoculated FRhK-4 cells, Figures 35 to 40

Picornavirus-like particles, approximately 23 nm in diameter, were found in thin sections of FRhK-4 cells examined 55, 107 or 128 days after inoculation with HAV (Figs 35-40). Very small numbers of such virus-like particles were detected in about 1% of the monolayer cells 55 (Fig 35) or 107 days after inoculation, and in larger numbers in spontaneously detached cells harvested at days 107 or 128 (Figs 36 & 38-40). Picornavirus-like particles could not be found in any of the

control, uninoculated FRhK-4 cells examined though variable numbers of mycoplasmas (Fig 37) were seen in control and HAV-inoculated cultures.

Picornavirus-like particles were observed in the cytoplasm of intact (Figs 35 & 36) as well as degenerate (Figs 38-40) cells, and were usually associated with autophagic vacuoles (Figs 35 & 36) or cytoplasmic membranes (Figs 38-40). Such picornavirus-like particles contained electron-opaque or -translucent cores (Figs 35b, 36c, 38b, 39b & 40b) and were sometimes completely enveloped by membrane (Fig 40).

3.14. Virus-like particles in thin sections of HAV-inoculated FRhK-4 cells, Figures 41 to 44

Also present in degenerate, spontaneously detached FRhK-4 cells, fixed 107 or 128 days after inoculation with HAV, there were rare arrays of roughly spherical virus-like particles (Figs 41-44). Many of these particles, which were approximately 39 nm in diameter, contained small irregular, and often eccentric, core-like structures (Figs 41-44). Occasionally "radial spokes" could be observed (Fig 43).

3.15. IEM: control virus morphology, Figures 45 to 48

The appearance of polyomavirions in Figures 45 to 48 is characteristic of the SV40, SA12 and polyoma virus antigens used for IEM. Virions were "clean" but occasionally associated with membranes (Fig 45); small clumps were often found (Figs 46-48). Sometimes fine strands were seen between the virus particles (Fig 47) but antibody was not identified. "Mini" polyomavirus particles (Figs 46 & 48), filaments, amorphous substance and free capsomers (Fig 47 & 48) and small round particles (Fig 45) were observed.

3.16. IEM: control viruses with homologous antisera, Figures 49 to 52

Figures 49 to 52 illustrate the range of antibody detected in IEM reactions between control antigens and different dilutions of their homologous antisera. Dense antibody coating of SV40 (Figs 49 & 50), SA12 and polyoma virus was obtained when high concentrations of antisera were used. The appearance of virus particles clumped by very small amounts of antibody is shown in Figures 51 and 52. Vacuoles (Fig 50), aberrant particles (Fig 52), filaments and "mini" particles could be found in virus-antibody aggregates.

3.17. IEM: identification of FRKV, Figures 53 to 62, Tables 3.1 and 3.2

Because free virus from culture fluids containing bovine sera was frequently antibody-coated (Figs 7, 8, 12, 14, 18 & 19), only virus from detached cells, or from cultures maintained on Iscove's serum-free medium or medium containing equine serum, was used in IEM tests. Sufficient virus could not be obtained from FRhK-6 cultures, so for some tests FRK6V was grown in CK17 cells. CK8 and CK26 cultures were used to grow WRSV.

Since polyomavirions had been detected in uninoculated CK17 culture after prolonged incubation (Fig 24), antigens for IEM were only harvested if, after extensive searching, virus could not be found in high speed pellets of fluids from control CK17, CK8 or CK26 cultures.

Each batch of antigen prepared was examined before use to confirm that antibody could not be found; the appearance of the virions illustrated in Figures 4 to 6, 9 to 11, 25 and 26 is representative of those in virus suspensions used for IEM.

Despite ultrasonication, groups of polyomaviruses occurred in all preparations (Figs 4, 5, 23, 25 & 46-48) thus the presence of aggregates of virus after incubation with test sera could not be interpreted unequivocally as the result of interaction between virus and antibody. IEM results were therefore only scored positive if virus particles were surrounded by a moderate or dense coating of antibody molecules. Since the amorphous substance attached to some virus particles made identification of sparse antibody difficult, no attempt was made to quantify the amount of antibody present by further dilution of sera.

Dense antibody coating of FRK4V (Figs 53-56), FRK6V (Figs 57-59) and WRSV (Figs 60 & 61) was observed with several bovine sera and both STMV-specific antisera 75/176 and 81/239 (Table 3.1). All virions in such preparations were coated with large amounts of antibody except on two occasions when the particles were membrane-bound (Fig 62) or associated with fragments of membrane (Fig 59). Amorphous substance was found near (Figs 53 & 56), attached to (Fig 54) or incorporated into (Figs 59 & 60) antibody-virus aggregates. "Mini" particles, vacuoles and filaments could be found clumped with normal virions (Fig 59). Dense or moderately dense antibody coating of FRK4V, FRK6V and WRSV was observed with the CMKV-specific antiserum and moderate amounts of antibody were found in two fetal bovine sera (Table 3.1).

Equivocal results were obtained when fetal bovine serum 27/5 and one of the rabbit FRKV antisera were tested in IEM with FRK4V: there was an increase in the number and size of aggregates of virus, with small amounts of antibody present on the surface of the particles, but dense antibody coating was not observed. Antibody against FRK4V could not be found in either of the other rabbit anti-FRKV sera, nor in the rhesus STMV antiserum.

Table 3.1. IEM: FRK4V, FRK6V and WRSV with various bovine sera, STMV antisera 75/176 and 81/239 and the CMKV antiserum

Serum		Diln	FRK4V	FRK6V	WRSV
fetal bovine sera	11/3	1/10	-	-	-
	24/8	1/10	-	-	-
	18/3	1/10	++	++	++
	14/10	1/10	++	++	nt
newborn bovine sera	17/8	1/10	+++	+++	+++
	22/6	1/10	+++	+++	++
	21/6	1/10	+++	++	+++
adult bovine serum	30/3	1/10	+++	++	++++
STMV rabbit antisera	75/176	1/5	+++	+++	nt
		1/10	+++	+++	+++
		1/20	nt	++	+++
	81/239	1/5	++++	nt	nt
		1/10	+++	+++	+++
		1/20	+++	+++	+++
CMKV monkey antiserum		1/10	+++	+++	++

FRK4V extracted from spontaneously detached FRhK-4 cells or cultures on Iscove's serum-free medium

FRK6V from spontaneously detached FRhK-6 cells, FRhK-6 cultures on Iscove's medium, or spontaneously detached CK17 cells 39 days after inoculation with FRK6V

WRSV grown in CK8 or CK26 cells with medium containing equine serum

STMV 81/239 antiserum was known to react with STMV in IEM (K V Shah, personal communication)

CMKV antiserum was known to react with CMKV in IF (C J A Sol, personal communication)

- = antibody not detected; ++ to ++++ = moderate amounts to very dense antibody; nt = not tested

Antibody could not be found when FRK4V or FRK6V was treated with specific antisera against the primate polyomaviruses SV40, SA12, JCV or BKV (Table 3.2). Antibodies against SV40 were not detected in newborn bovine sera 17/8 or 21/6, STMV antisera 75/176 or 81/239, nor against polyoma virus in newborn bovine sera 21/6 or 22/6.

Table 3.2. IEM: FRK4V and FRK6V with primate polyomavirus antisera

Antiserum	Diln	Homologous virus	FRK4V	FRK6V
SV40 calf	1/25	+++	-	-
SA12 rabbit	1/10	+++	-	-
JCV guinea pig	1/15	+++	-	-
JCV human	1/10	+++	-	-
BKV guinea pig	1/15	+++	-	-

FRK4V extracted from spontaneously detached FRhK-4 cells or cultures on Iscove's serum-free medium

FRK6V from spontaneously detached FRhK-6 cells, FRhK-6 cultures on Iscove's medium, or spontaneously detached CK17 cells 39 days after inoculation with FRK6V

JCV and BKV antisera were known to react with JCV and BKV in IEM (A M Field, personal communication)

- = antibody not detected; +++ = dense antibody

3.18. Immunofluorescence

Immunofluorescence was used to detect the presence of antigen in FRhK-4 cells using an anti-FRK4V rabbit serum (S D Gardner). Characteristic polyomavirus fluorescent staining of nuclei with sparing of the nucleoli was detected. Usually cytopathic changes were minimal and approximately 10% of the cells gave specific nuclear fluorescence. In an occasional coverslip extensive spontaneous degeneration of the monolayer, which appeared to be specific, was observed; in these cultures fluorescence was strongly positive and polyomavirions with moderate to dense antibody

coating were observed in high speed pellets of culture fluid. When FRK4V was grown in CK cells, about 75% of nuclei gave positive fluorescence. Fluorescent staining of control, uninoculated CK cells was not detected.

Positive fluorescent staining of nuclei was observed when FRK4V-infected cultures were reacted with the rhesus monkey STMV antiserum, which was known to contain SV40 group antibody, with both rabbit STMV antisera and with the monkey CMKV antiserum. Positive fluorescence was also observed when WRSV-infected cells were treated with STMV antiserum 75/176. Specific nuclear fluorescence was not detected when specific rabbit antisera to BKV, JCV, SV40 or SA12 were used, or with human sera known to contain antibodies against BKV or JCV.

3.19. Morphology of virus in CIE precipitin lines formed between FRK4V and various sera, Figures 63 to 66

Initially, FRK4V extracted from FRhK-4 cultures for IEM was used as antigen in CIE tests (J V Parry). Newborn bovine serum 22/6, which had been shown by IEM to contain anti-FRKV (Fig 58 & Table 3.1), was designated the antibody-positive control and was included in every gel. Dense antibody coating was characteristic of the polyomavirions in the CIE precipitin lines formed between FRK4V and both of the preparations of IgG purified from newborn bovine serum 22/6. Identical reactions were obtained from newborn bovine serum 22/6 that had been treated with RDE to remove non-specific inhibitors (Fig 63).

Virus precipitation was also detected by CIE between FRK4V and the following: two additional RDE-treated newborn bovine sera; one fetal bovine serum; three newborn bovine sera (including 22/6); and individual

sera from five cattle aged between one week and adult (Figs 64 & 65). Densely antibody-coated virions were seen in all of these gel lines.

The antibody in CIE virus precipitates, whether these were produced by purified bovine IgG, RDE-treated sera (Fig 63) or individual bovine sera (Figs 64 & 65), was indistinguishable from both "naturally" occurring antibody molecules (Figs 7, 14, 18 & 19) and antibody observed in IEM reactions (Figs 2, 3, 49, 50 & 53-61).

With two additional fetal bovine sera and two of the rabbit FRKV antisera, precipitin lines contained virus clumped with only small amounts of antibody, as well as some polyomavirus particles on which antibody could not be identified (Fig 66).

The third rabbit anti-FRKV serum, seven newborn bovine sera and 16 of 27 (59%) fetal bovine sera tested by CIE contained anti-FRKV; a further five of 27 (19%) fetal bovine sera gave equivocal results (J V Parry). These precipitin lines were not examined by EM.

Subsequently, FRKV grown in CK cells was used in CIE for serological studies (J V Parry). Antibody could not be detected in any of 45 sera from rhesus monkeys, nor in any of 97 human blood donor sera. When 353 sera from cattle of all ages between one month and 12 years were tested, anti-FRKV was found in 170 (48%); 12 (3%) more sera gave equivocal results.

3.20. IEM: polyomavirus from uninoculated LLC-MK2 culture, Figures 67-70 and Table 3.3

Distinct cytopathic effects were not observed in uninoculated monolayers of LLC-MK2 and MA104 cells in use at the Virology Laboratory at

St Thomas's Hospital, but the presence of a few vacuolated cells prompted examination by EM (B M Totterdell). Polyomavirus particles were seen in preparations of culture fluid from both cell lines after eight weeks of incubation.

Moderate amounts of antibody were attached to approximately 75% of the polyomavirions in the high speed pellet of culture fluid from uninoculated LLC-MK2 cells with only traces of antibody on the remaining virions (Figs 67 & 68). Similar results were obtained when this preparation was tested by IEM with human polyomavirus antisera (Table 3.3). In IEM tests with goat anti-bovine IgG, the same proportion of virus particles was surrounded by particularly dense antibody (Figs 69 & 70). However, all virions were densely coated with antibody when LLC-MK2V was mixed with fetal or newborn bovine sera or with STMV antiserum 81/239 (Table 3.3).

Table 3.3. IEM: LLC-MK2V with various sera and goat anti-bovine IgG

Serum		Diln	Virus with ab	Antibody
BKV guinea pig antiserum		1/15	75%	++
JCV guinea pig antiserum		1/15	75%	++
goat anti-bovine IgG		1/20	75%	++++
fetal bovine serum	14/10	1/10	100%	+++
newborn bovine sera	21/6	1/10	100%	+++
	17/8	1/10	100%	+++
STMV rabbit antiserum	81/239	1/20	100%	+++

LLC-MK2V from high speed pellet of fluid from uninoculated LLC-MK2 culture; medium contained 2% fetal bovine serum

++ to ++++ = moderate to very dense antibody

Small round particles (Fig 67), "mini" virions (Fig 70) and occasional plumes or trails (Fig 68) also were seen in LLC-MK2V preparations. Virus particles could not be found in high speed pellets of either the fetal bovine serum or the trypsin used.

The same batch of fetal bovine serum had been used to grow and maintain both LLC-MK2 and MA104 cell lines. Cryopreserved cells with a similar passage history were later grown using a different batch of fetal bovine serum, but polyomavirions were not detected by EM in either cell line (B M Totterdell). Subsequently, RIA tests showed that the MA104 polyomavirus isolate was antigenically similar to FRKV (J V Parry).

3.21. Polyomaviruses detected in uninoculated CK cultures exposed to fetal bovine serum U720201D, Figures 71 to 75, Tables 3.4 and 3.5

3.21.1. Calf kidney cultures CK8 to CK28, Figures 71 to 73, Table 3.4

Polyomavirions were found in 18 of the 19 batches of calf kidney cells, CK8 to CK28, received from Weybridge; virus was not detected in CK23 which degenerated after six weeks. The same fetal bovine serum, U720201D, had been used in both growth and maintenance media for all 19 batches of cells. However, virus particles could not be found in high speed pellets of this serum. In some cultures very small numbers of polyomavirus particles with moderate to dense antibody coating were observed (Table 3.4), but in harvests which contained moderate to large numbers of particles, antibody was rarely seen (Figs 71-73).

"Normal" polyomavirions (Fig 71), filaments (Fig 72), small round particles (Fig 73) were observed. There were numerous "mini mini" polyomavirus particles, approximately 21 nm in diameter, which were occasionally associated (Fig 72) with the variable amounts of amorphous

substance that were present (Figs 71-73).

The period of incubation before virus was detected varied (Table 3.4). The earliest culture fluids found to contain polyomavirions were collected from CK26 and CK14 after five and seven weeks of incubation; these batches had been negative at weeks four and six respectively. The latest EM negative samples were collected from CK15 and CK18 at weeks 16 and 17; these two cultures were positive at weeks 18 and 25.

Table 3.4. Harvests from uninoculated calf kidney cultures in which fewer than ten polyomavirus particles were detected

Culture	CK8	CK13	CK14		CK15	CK20*
Number of virions	7	3	6	3	5	3
Antibody	(+)	+++	+++	+++	++	++
Weeks of incubation	17	18	7	9	18	9

High speed pellets of culture fluids; media contained 2% fetal bovine serum U720201D

(+) = probable antibody but could be amorphous substance; ++ to +++ = moderate to dense antibody

*CK20 degenerated at week 9

Retrospective RIA (J V Parry) testing of the latest fluid from each CK culture demonstrated FRK antigen in all but three. However, in these three RIA-negative samples small numbers of polyomavirions had been found by EM; in one sample virus was densely coated with antibody.

3.21.2. Calf kidney cultures CK29 to CK42, Figures 74 and 75, Table 3.5

Calf kidney cells CK29 and CK31 to CK33 were exposed to media containing 10% or 2% fetal bovine serum U720201D for two to six weeks (Table 3.5). Maintenance medium for CK29/2 contained equine serum, but

gamma-irradiated fetal bovine serum was used in all other media for CK29 to CK42 cultures.

Table 3.5. Exposure of CK29 to CK33 cultures to the suspect fetal bovine serum U720201D and subsequent detection of polyomaviruses

Culture	U720201D exposure	EM observations
CK29/1	10% in GM, 2 weeks	9 +++ antibody-coated virions at week 8
CK29/2	10% in GM, 2 weeks	4 virions, no antibody at week 8
CK30	none	virus not detected up to week 12
CK31/1	2% in MM, 4 weeks	*** virus, no antibody at week 15
CK31/2	none	virus not detected up to week 19
CK32/1	2% in MM, 2 weeks	virus not detected up to week 18
CK32/2	none	virus not detected up to week 18
CK33/1	2% in MM, 6 weeks	7 +++ antibody-coated virions at week 11*
CK33/2	none	virus not detected up to week 17

GM = growth medium; MM = maintenance medium

Gamma-irradiated fetal bovine serum was used in all other GM and MM, except CK29/2 MM which contained 2% equine serum

*** virus = large numbers of virions; +++ antibody = dense antibody

*CK33/1 culture died at week 11; this harvest was later found to be RIA-negative

Variable numbers of polyomavirions were seen in all of the cultures exposed to the suspect serum except CK32/1 which had been in contact with 2% serum U720201D for the shortest period (Table 3.5). Virus particles could not be found by EM in any of the CK30 to CK42 cultures that had been exposed only to gamma-irradiated serum, but FRKV antigen

was subsequently detected by RIA (J V Parry) in one of these 13 batches of cells.

3.22. IEM: influence of serum in CK29 culture media on amount of antibody detected, Figures 74 to 77, Tables 3.5 and 3.6

After two weeks on growth medium containing fetal bovine serum U720201D the CK29 culture was divided: maintenance medium for CK29/1 cells contained gamma-irradiated fetal bovine serum, that for CK29/2 contained equine serum. Very small numbers of polyomavirions were observed in both cultures after incubation for eight weeks (Table 3.5), virus not having been detected in either the preceding week.

By week ten, when larger numbers of particles were found in high speed pellets of culture fluids from both batches of cells, approximately 30% of CK29/1 virions were moderately coated with antibody (Fig 74) the remainder having little or no antibody attached. Although amorphous substance was sometimes attached to CK29/2 virus particles, antibody could not be identified (Fig 75). "Normal" polyomavirions, "mini" particles, large numbers of small roundish particles, filaments and narrow filaments were found (Figs 74 & 75). Free capsomers were occasionally associated with amorphous substance (Fig 75) and slightly electron-translucent plumes or trails were also observed (Fig 74).

The results of CK29/1V and CK29/2V IEM with various bovine sera and both STMV antisera are presented in Table 3.6. In all instances more antibody was observed with CK29/1V (Figs 75 & 76).

Table 3.6. IEM: effect of serum in culture medium on antibody observed

Serum		Diln	CK29/1V	CK29/2V
fetal bovine sera	11/3	1/10	++/-	-
	14/10	1/10	+++	++
newborn bovine sera	17/8	1/10	++++	++
	21/6	1/10	+++	++
STMV rabbit antisera	75/176	1/20	+++	++
		1/50	++	+
	81/239	1/20	++++	+++

CK29/1 medium contained 2% fetal bovine serum; CK29/2 medium contained 2% equine serum

++/- = some virions with moderate antibody, some with little or none

- = antibody not detected; + to ++++ = small amounts to very dense antibody

3.23. IEM: four other CK polyomavirus isolates, Figures 78 to 84 and Table 3.7

Harvests containing large numbers of virus particles, but in which antibody was not identified, were used in IEM experiments and the results are presented in Table 3.7.

Antibodies against the four CK polyomaviruses were either not detected or found only in small to moderate amounts in two newborn bovine sera and both rabbit STMV antisera (Table 3.7a, Figs 78-81). An exception was the very dense antibody coating of CK12V observed when STMV antiserum 81/239 was used at a dilution of 1/20 (Fig 82). These four sera, however, contained large amounts of antibody against CK29/1V (Table 3.6, Figs 83 & 84), and against FRK4V, FRK6V and WRSV (Table

3.7a). In particular compare the antibody in Figure 78 with 54, 60 and 61; Figures 79 and 81 with 53, 57 and 58 which gave identical results to newborn bovine serum 21/6; and Figure 80 with 55, 56 and 59.

Table 3.7a. IEM: various viruses with newborn bovine sera and STMV antisera - amounts of antibody observed

Sera diluted 1/10		FRK4V	FRK6V	WRSV	CK8V	CK9V	CK10V	CK12V
newborn bovine sera	17/8	+++	+++	+++	-	-	++	-
	21/6	+++	++	+++	+	+	++	-
STMV antisera	75/176	+++	+++	+++	++	++	++	+
	81/239	+++	+++	+++	++	+	+++	++

- = antibody not detected; + to ++++ = small amounts to very dense antibody

Table 3.7b. IEM: various viruses with newborn bovine sera and STMV antisera - numbers of virus particles found

Sera diluted 1/10		FRK4V	FRK6V	WRSV	CK8V	CK9V	CK10V	CK12V
newborn bovine sera	17/8	**	**	*	*	*	*	*
	21/6	*	*	**	*	*	*	*
STMV antisera	75/176	*	*	*	***	***	**	***
	81/239	**	*	*	***	**	**	***

Virus particles seen: * = 10-149; ** = 150-249; *** >= 250. Each grid was examined for 20 minutes

FRK4V extracted from spontaneously detached FRhK-4 cells or cultures on Iscove's serum-free medium

FRK6V from spontaneously detached FRhK-6 cells, FRhK-6 cultures on Iscove's medium, or spontaneously detached CK17 cells 39 days after inoculation with FRK6V

WRSV grown in CK8 or CK26 cells with medium containing equine serum

CK viruses from high speed pellets of CK8, CK9, CK10 and CK12 culture fluids; media contained 2% fetal bovine serum U720201D

The numbers of polyomavirions found in IEM preparations are shown in Table 3.7b. With newborn bovine sera 17/8 and 21/6 approximately comparable numbers of all virions were seen. When the two rabbit anti-STMV sera were used, more CK8, CK9, CK10 and CK12 virus particles were found than when those sera were used in IEM with FRK4V, FRK6V and WRSV.

3.24. The use of antibody-coated grids (ACG), Figures 85 to 88 and Tables 3.8 to 3.10

Very small numbers of antibody-coated virus particles were frequently encountered in harvests from FRhK-4 (Section 3.3), FRhK-6 (Section 3.4) and CK (Tables 3.4 & 3.5) cells. Moreover, only one such virion could be found in DBS-FRhL-2 cultures (Section 3.5). When such small numbers of virions were found, counts could not be reproduced when the same specimen was re-examined. The difficulty was clearly demonstrated when several aliquots of culture fluids from CK14 at weeks seven and nine were centrifuged. The numbers of particles observed varied not only from one preparation to the next, but also when the same grids were subsequently re-examined (Table 3.8).

Goat anti-bovine (GAB) IgG was shown to react with the antibody attached to LLC-MK2V (Figs 67-70). In an attempt to improve the detection rate of antibody-coated virions, the use of GAB IgG to coat grids was compared with that of rabbit (RAB) anti-bovine IgG. Densely antibody-coated polyomavirions, filaments "mini" and "mini mini" spherical particles were found in all preparations (Figs 85-88). With both IgG preparations more virus particles were found on some of the coated grids (Table 3.9) than the seven virions that were observed on the control grid.

Table 3.8. Numbers of antibody-coated polyomavirions found in aliquots of two CK14 harvests after centrifugation and re-examination of grids

High speed pellets	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
Initial examination	6	-	-	3	-	3	10	15	7	33
Repeat examination	-	nt	10	3	nt	3	-	13	nt	31

P1 to P3 culture fluid at week 7, P4 to P9 clarified culture fluid at week 9; media contained 2% fetal bovine serum U720201D. P10 = P1 to P9 pooled and re-centrifuged

- = virus particles could not be found. Each grid was examined for 20 minutes

Table 3.9. ACG: numbers of CK14 polyomavirions found using goat or rabbit anti-bovine IgG at various dilutions

Dilutions	1/100	1/500	1/1000	1/1000*
goat anti-bovine IgG	42	6	29	-
rabbit anti-bovine IgG	6	37	13	7

* BPA added to 1/1000 dilutions of GAB and RAB; - = virus particles could not be found

Control grid, without IgG coating: 7 polyomavirus particles were seen

In a series of replicate tests using GAB IgG, there was little or no increase in virus detected on the coated grids compared with non-coated, normally prepared control grids (Table 3.10). Virions could not be found in any of the DBS-FRhL-2 culture fluids prepared by this method.

Table 3.10. ACG: numbers of CK14 polyomavirions found in replicate tests using goat anti-bovine IgG at various dilutions

goat anti-bovine IgG	1/100	1/500	1/1000
Initial ACG tests	12	49	36
Repeat 1 ACG tests	78	30	73
Repeat 2 ACG tests	33	34	27
Repeat 3 ACG tests	37	24	73

Control grid, without IgG coating: 33 polyomavirus particles were seen (P10, Table 3.8)
CK14 virus: pool of pellets P1 to P9 (Table 3.8); all grids on antigen preparation for 1h at +4°C

3.25. Non-polyomavirus particles, Figures 89 to 92

Non-polyomavirus particles, some of which are illustrated in Figures 89 to 92, were present in many preparations. Large indistinct structures, about 73 nm in diameter, were frequently observed (Fig 89). At first glance such structures were vaguely similar to very densely antibody-coated polyomavirions but closer examination did not reveal any evidence of polyomavirus morphology. Roughly elliptical particles, approximately 67 nm by 38 nm, were rarely found and on one occasion were seen in chains (Fig 90). Large spherical structures, which measured about 56 nm (Fig 91), and bacteriophages (Fig 92) were present in some preparations.

3.26. The association of capsomers with membranes, Figures 93 to 96

"Free" polyomavirus capsomers, approximately 6.2 to 7.4 nm in diameter, were observed in preparations not only of FRKV and CK isolates but also of SV40 and polyoma virus. Capsomers were associated with amorphous substance (Figs 47, 48 & 75), with disintegrating virions or filaments

(Fig 66) and with membranes (Figs 93-96). On rare occasions, closely packed capsomers were found in paracrystalline array (Fig 96).

3.27. Electron-translucent trails or plumes, Figures 97 to 100

Indistinct structures resembling plumes or trails of slightly electron-translucent substance were observed adjacent to HAV (Fig 2) and polyomavirus particles (Figs 68, 74 & 97-100). The virions with which these structures were associated were antibody-coated (Figs 2, 68, 74 & 97) and/or damaged (Figs 74, 98-100).

3.28. Small, roundish, featureless particles associated with polyomavirions, Figures 101 to 108

Small, roundish, featureless particles (SRP) were frequently associated with polyomavirions; their shape, size and number varied considerably (Figs 101-108). In rare instances such particles appeared to be budding from membranes (Fig 105) or emerging from polyomavirions (Figs 107 & 108). Small round particles were present in many of the cell lines: FRhK-4 (Figs 6, 13, 101 & 102), FRhK-6 (Figs 10, 11 & 103), CK (Figs 22, 73, 74, 104 & 108), Vero (Fig 45) and LLC-MK2 (Fig 67). Preparations in which SRP were found included low speed pellets of spontaneously detached cells and high speed pellets of culture fluids or clarified culture fluids.

3.29. The association between amorphous substance and papovavirions, Figures 109-114

Papovavirus particles were sometimes found "embedded" in amorphous substance (Figs 109-111). On rare occasions virions appeared to be

forming within or emerging from (Figs 112-114) large areas of amorphous substance. Such observations applied to polyomavirus isolated from calf kidney cultures (Figs 109-111), SV40 in Vero cells (Fig 112), FRK4V grown in HEK culture (Fig 113) and to human papillomavirus (Fig 114). Fragments (Figs 110, 113 & 114) and larger pieces (Figs 111 & 112) of membrane were observed with such papovavirus-associated amorphous substance.

4. MORPHOLOGICAL OBSERVATIONS AND DISCUSSION

4.1. Morphology of polyomaviruses and associated structures

4.1.1. Morphology of polyomaviruses

The morphological features in thin section of polyomaviruses and a few papillomaviruses are summarized in the Appendix, Table A.1. Their features when prepared by other methods are shown in the Appendix, Table A.2.

Filamentous forms and the association of polyomavirions with cell membranes have frequently been seen in thin sections and negatively stained preparations (Tables A.1 & A.2). Thus the presence of polyomavirus filaments and membrane-associated virions in preparations of FRKV was not considered unusual. Paracrystalline arrays of FRKV were rare but this may simply reflect the small proportion of FRhK-4 cells infected.

"Mini" virus particles 30 to 38 nm and "mini mini" particles 17 to 22 nm in diameter of polyoma virus (Mattern, Takemoto & DeLeva, 1967), SV40 (Koch et al, 1967; Anderer et al, 1967) and other polyomaviruses have been described (Table A.2) and were common in preparations of FRKV. The three sizes of polyoma virus particles 42, 38 and 22 nm are thought to consist of 72, 32 and 12 capsomers respectively (Mattern et al, 1967).

4.1.2. "Free" polyomavirus capsomers

There have been several descriptions or illustrations of "free" papovavirus capsomers (Table A.2), usually associated with the disintegration of virions. Papillomavirus capsomers are 8 nm (Noyes, 1964; Smith et al, 1965) to 10 nm (Breedis, Berwick & Anderson, 1962; Finch & Klug, 1965; Klug & Finch, 1965) in diameter. The approximate size of "free" FRKV, SV40 and polyoma virus capsomers, 6.2 to 7.4 nm, in

the present study is comparable to the 6.5 nm reported for capsomers of polyomavirions purified from PML brain (Schwerdt et al, 1966) and slightly larger than the 5 nm of capsomers assembled into virions of polyoma (Wildy et al, 1960; Finch, 1974), RKV (Chambers et al, 1966), from PML brain (Howatson, Nagai & ZuRhein, 1965; Albert & ZuRhein, 1974), JCV and BKV (Albert & ZuRhein, 1974).

4.1.3. Possible papovavirus "factory" areas

Moderately electron dense, granular or finely fibrillar "factory" areas or viroplasm, in which virions are assembled, have been described in numerous thin section studies of virus morphogenesis including studies of ECHO virus 9 (Rifkind et al, 1961), poliovirus types 1 and 2 (Mayor & Jordan, 1962; Dales et al, 1965), simian foamy and bovine syncytial viruses (Dermott, Clarke & Samuels, 1971) rotavirus (Holmes et al, 1975; Suzuki et al, 1984; Poruchynsky, Maass & Atkinson, 1991) and poxviruses, for example vaccinia (Stern & Dales, 1976).

There have, however, been few examples of negatively stained viroplasm. This is not surprising since the identification of such morphologically undifferentiated areas in negatively stained preparations is undoubtedly more difficult than in thin sections in which their relatively uniform texture distinguishes them from the surrounding cytoplasmic matrix.

A notable exception was a study by Horne and Nagington (1959) who described the assembly of virions from subunits within a containing membrane in negatively stained preparations of poliovirus-infected HeLa cells. Recognizable virus particles, in various stages of formation, were frequently observed in well defined bodies which resemble the possible "factory" areas in Figures 109 to 114. Subunits were visible

in poliovirus particles and the surrounding stroma; incomplete virus linked to undifferentiated subunit stroma was also seen (Horne & Nagington, 1959).

In negatively stained mounts of whole virus-infected cells, bluetongue virions appear to be assembling in and emerging from areas of differently "textured" cytoplasm (Hyatt, Eaton & Brookes, 1989; Hyatt & Eaton, 1990). Such regions of cytoplasm might represent viroplasm and resemble possible "factory" areas in Figures 109 to 114.

The amorphous substance, which was frequently attached to or associated with FRKV and is present in preparations of many other polyomavirions (Table A.2), is presumably cellular in origin and may represent viroplasm. SV40, FRKV and papillomavirions (Figs 112, 113 & 114) occasionally seemed to be forming within or emerging from such amorphous substance.

4.1.4. Self-assembly of polyoma virus and SV40

The capsid of polyoma virus is self-assembling from units of capsomer size (Friedmann, 1971) and assembly of SV40 is a random process from pools of unassembled viral capsid protein (Ozer & Tegtmeyer, 1972) the size of which increases during the late phase of virus replication (Girard et al, 1973). The presence of capsomers in amorphous substance in preparations of SV40 and CK29/2V (Figs 47, 48 & 75) suggests that amorphous substance could be part of such an assembly process.

4.1.5. Plumes or trails of electron-translucent substance

On heating, bacteriophage ϕX174 extruded strands which were thought to consist of DNA irregularly coated with protein (Maclean & Hall, 1962). Such strands resemble the plumes of electron-translucent substance

associated with FRKV (Figs 68, 74 & 97-100) and HAV (Fig 2). A similar structure associated with a damaged polyoma virion is illustrated by Crawford, Crawford and Watson (1962).

Empty capsids and fibrillar structures have been observed after various treatments of poliovirus (Van Elsen, Boeye & Teuchy, 1968) and heat degradation of poliovirus and rhinovirus led to the extrusion of ribonucleoprotein strands (McGregor & Mayor, 1968; 1971). Brief heating of these viruses resulted in the formation of diffuse amorphous cores resembling "puffballs" which became progressively extended until long strands were observed suggesting that RNA is first released then unfolded (McGregor & Mayor, 1971). The two SRP which appear to be emerging from polyomavirions (Figs 107 & 108) were similar to poliovirus and rhinovirus amorphous cores and might just be an intermediate stage in the formation of plumes, but this is considered unlikely.

Although the trails of electron-translucent substance could be preparation artefacts, it is possible that they represent nucleic acid released from antibody-coated or spontaneously damaged HAV particles and polyomavirions. Morphological studies of deliberately damaged virions might help to elucidate the nature of such structures.

4.1.6. Small roundish particles (SRP)

The small, roundish, featureless particles attached to FRKV in many preparations (including Figs 101-108) were similar to particles that have been illustrated in suspensions of many polyomaviruses (Table A.2). They were variable in size and shape and did not resemble the picornavirus-like particles in a porcine kidney cell line that was infected with the swine polyomavirus, SPV (Tischer, Rasch & Tochtermann,

1974); the picornavirus-like particles have subsequently been identified as porcine circovirus (Tischer et al, 1982). However, the SRP in the PK-15 (Tischer et al, 1974) and CCL 33 (Tumilowicz, Hung & Kramarsky, 1979) cell lines are morphologically indistinguishable from those in FRKV preparations (Figs 101-106) and are thought to be components of bovine serum (Tumilowicz et al, 1979).

Negatively stained preparations of lipid particles (6 to 20 nm in diameter) from rough endoplasmic reticulum (RER) and serum very low density lipoproteins (14 to 40 nm in diameter) (Banerjee & Redman, 1984) are like the FRKV-associated SRP (Figs 101-106), but are not quite as "solid-looking" and more strongly resemble the artefact in serum which looks like hepatitis B surface antigen (HBsAg) (Field, 1982).

However, FRKV-associated SRP were found in preparations of detached cells (Figs 6, 10, 11, 101 & 106), many of which had been washed with PBS before being sonicated, and therefore are unlikely to have originated from the calf serum used in the medium.

4.2. Morphology of miscellaneous structures

4.2.1. Virus-like particles in thin sections of FRhK-4 cells

There are several possible explanations for the virus-like particles found in HAV-infected FRhK-4 cells (Figs 41-44). These roughly spherical particles did not appear involved in the morphogenesis of either HAV or polyomavirions but were morphologically distinct structures present in sufficient numbers (Figs 41-44) to suggest that they were not artefacts of preparation.

Thin sections of simian foamy virus in the perinuclear cistern of monkey kidney cells (Clarke, Gay & Attridge, 1969) and some illustrations of bovine syncytial and simian foamy virions in BHK-21 cells (Dermott et al, 1971) resembled the particles in Figures 41 to 44. However, the budding particles, surface spikes and free cores characteristic of these and related retroviruses could not be found in FRhK-4 cells.

Although some of the immature rotavirus particles in thin sections of duodenal biopsies (Holmes et al, 1975) and cell cultures (Suzuki et al, 1984; Poruchynsky et al, 1991) are similar to the particles in Figures 41 to 44, nothing even remotely like rotavirus was seen in negatively stained preparations. Furthermore, the clearly defined dense cores, areas of viroplasm, budding and mature virions that are present in rotavirus-infected cells could not be found in FRhK-4 cells.

Semliki Forest virions, approximately 60 nm in diameter, in thin sections of BHK cells (Peranen & Kaariainen, 1991) most closely resemble the virus-like particles in FRhK-4 cells (Figs 41-44). However, unlike the particles in Figures 41 to 44, Semliki Forest virions are rounder, generally have a more distinct cores and are aligned along the inner surface of cytoplasmic vacuoles.

Thin sections of rubella virus in Vero cells (Lee, Marshall & Bowden, 1992) revealed cytoplasmic vacuoles containing 60 nm vesicles, morphologically distinct from rubella virions, which were located on the membrane surrounding the replication complex and often displayed an irregular dense core (Lee et al, 1992). Some of these vesicles are vaguely similar to the virus-like particles in FRhK-4 cells (Figs 41-44) except that budding particles were not found and the rubella virus-associated vesicles were aligned along membranes.

Annulate lamellae are cytoplasmic organelles composed of stacked sheets of membrane containing hexagonally packed pores or annuli that are structurally indistinguishable from nuclear pores (Stafstrom & Staehelin 1984). If the plane of section is favourable, annuli appear as 110 nm particles of high electron-opacity without a limiting membrane surrounded by moderately electron-opaque substance (Nakayama et al, 1977). Thus *en face* views of annulate lamellae do not account for the virus-like particles in Figures 41 to 44 since these structures have a definite edge and vertical sections through stacks of membrane could not be found in FRhK-4 cells. Furthermore, the virus-like particles in Figures 41 to 44 do not look like tangentionally sectioned nuclear pores (Richmond, unpublished observations; Field, 1982).

Cross sections through the tubular aggregates of the rhesus monkey ciliary body (Martins-Green & Roth, 1982) bear some resemblance to the strange virus-like particles (Figs 41-44) but there was no evidence of tubules in longitudinal section in FRhK-4 cells.

Polyhedral virus-like particles, approximately 39 nm in diameter, which are present in hyphae of the fungus *Lentinus edodes* are thought to belong to the reovirus group (Ushiyama & Nakai, 1982). The particles in Figures 41 to 44 resemble the arrays of empty fungal virus-like structures and those with partial cores, but not those with dense, mature cores (Ushiyama & Nakai, 1982) and, although hyphae were not observed, the presence of a fungal virus cannot be excluded.

R-type particles are usually spherical, but sometimes elongated or slightly oval, and vary in size from 85 to 130 nm. They have a dense core and radial spoke-like structures can sometimes be seen. The particles are frequently found within the nuclear envelope or in

cisternae of the endoplasmic reticulum (ER), often arranged in a chain-like order, and have been observed in hamster cells *in vitro* (Bernhard & Tournier, 1964; Compans et al, 1964; de Petris & Harvey, 1969; Shipman, Vander Weide & Ma, 1969; Anderson & Doane, 1972; Epstein & Fukuyama, 1973; Sindelar et al, 1983) and *in vivo* (Epstein & Fukuyama, 1970). R-type particles have also been seen in calf kidney (Mussgay, Reczko & Ahl, 1969) and gerbil (Yelle & Berthiaume, 1982) cells *in vitro*.

The R-type particles in BHK21-F (Compans et al, 1964) and gerbil cells (Yelle & Berthiaume, 1982), in which radial spokes are not particularly clear, are more like the particles in Figures 41 to 44 than R-type particles illustrated by others.

Although the structures considered bear a greater or lesser resemblance to the virus-like particles in FRhK-4 cells (Figs 41-44) none was considered convincing, thus the nature of these particles remains unresolved.

4.2.2. Large indistinct structures

Intracytoplasmic A-type particles of Mason-Pfizer, murine leukaemia and murine mammary tumour viruses in negatively stained whole cell mounts (Kramarsky, Sarkar & Moore, 1971) resemble the large indistinct particles shown in Figure 89. However, A-type particles could not be found in thin sections of FRhK-4 cells.

Negatively stained whole cell preparations have also been used to study the release of bluetongue virus, a member of the *Reovirus* family. Although non-enveloped and enveloped bluetongue virions (Hyatt et al, 1989) are similar to the large indistinct structures (Fig 89), reovirus-like particles were not observed in any of the negatively

stained preparations examined.

It is possible that the indistinct particles (Fig 89) might be coated vesicles, normal cell organelles, which are about 55 to 85 nm in diameter (Pearse, 1975; Benson et al, 1985). However, the outer portion of negatively stained coated vesicles show pentagonal and hexagonal features which were not seen in the indistinct structures in the present study.

The nature of these large indistinct particles also remains unresolved.

4.2.3. Elliptical particles

Bacteriophages have been isolated from fetal bovine serum used in cell culture (Merril et al, 1972; Chu et al, 1973) and the elongated roughly elliptical structures (Fig 90), which do not resemble previously described animal viruses, might be bacteriophage particles. They bear a vague resemblance to the negatively stained scaffold of bacteriophage T4 and *Sulfolobus shibatae* virus stained with UA is also somewhat similar but appears more "solid" (Wurtz, 1992).

However, illustrations of two elliptical particles identical to those in Figure 90 have been found in the literature: one in a mixture of RKV and human papillomavirus (Crawford & Follett, 1967), the other in a glutaraldehyde-fixed preparation of yellow fever virus (Madeley & Field, 1988). In neither of these publications was there comment about the elongated structures.

Elliptical particles are therefore present in preparations of various viruses grown in different cultures including calf kidney (Fig 90), rabbit kidney (Crawford & Follett, 1967) and Vero cells (Madeley & Field, 1988). They have also been found in other preparations

(Richmond, unpublished observations) and one common factor is probably the use of calf serum in the culture medium.

4.2.4. Non-polyomavirus particles

Although isolated nuclear pore complexes (Aebi et al, 1984) bear some silimarity to the non-polyomavirus particles in Figure 91, these structures look more like bacteriophages aggregated by immunoglobulin M (IgM). The large spherical particles are about the same size as bacteriophage heads (Fig 92) and are similar to the faecal bacteriophages agglutinated by a naturally occurring antibody (Flewett & Boxall, 1976).

4.3. Hepatitis A virus morphology

The morphology of negatively stained HAV grown in FRhK-4 culture was characteristic of picornaviruses and indistinguishable from virus passaged in marmosets. Antigens from both sources reacted identically in IEM with purified anti-HAV IgG. The size of the virions, approximately 27 nm, was the same as that previously reported for HAV (Feinstone et al, 1973; Provost et al, 1975; Siegl & Frosner, 1978; Flehmig, 1980).

Several features observed in thin sections of HAV-infected FRhK-4 cells were characteristic of picornavirus replication. Electron-opaque and electron-translucent picornavirus-like particles, found in HAV-inoculated but not in control cells, were indistinguishable from ECHO 9 virions (Rifkind et al, 1961). Chains and small clusters of particles resembled those of Coxsackie B5 virions (Nunez-Montiel et al, 1961) and vacuoles were found in spontaneously detached FRhK-4 cells but were not

pronounced in monolayer cells. Short linear arrays and randomly distributed particles, usually associated with membranes, were like those of ECHO 4 virus (Duffy et al, 1962) and the association of membranes with picornavirus-like particles in HAV-infected FRhK-4 cells was similar to that described in cells infected with poliovirus types 1 and 2 (Mayor & Jordan, 1962; Dales et al, 1965).

The size of picornavirus-like particles in HAV-infected FRhK-4 cells, approximately 23 nm in diameter, was similar to the 22 nm reported for ECHO 9 virions (Rifkind et al, 1961) and was between the 17 to 19 nm of Coxsackie B5 virus (Nunez-Montiel et al, 1961) and the 26 to 28 nm of poliovirus type 1 (Dales et al, 1965).

Based on their size, cytoplasmic development and morphology, which was the same as that of HAV in thin sections of marmoset (Huang, Lorenz & Gerety, 1979) and human (Shimizu et al, 1982) liver, it was concluded that the picornavirus-like particles in FRhK-4 cells were hepatitis A virions. The slightly smaller size in thin sections than the 27 nm of negatively stained virions might be due to shrinkage during fixation, dehydration and embedding of the specimens. Using monospecific antisera against N or H antigens of poliovirus type 1, Hummeler, Anderson and Brown (1962) found that anti-N agglutinated "full" virions, which contained RNA, whereas anti-H aggregated "empty" particles, which lacked RNA. The electron-opaque and electron-translucent particles found in thin sections probably correspond to the "full" and "empty" negatively stained virions and, like poliovirus type 1, electron-opaque and "full" particles may contain RNA.

The genomic and growth characteristics of HAV are so distinct that it has been suggested that the virus should not be classified as a typical

member of any of the four picornavirus genera (Ticehurst, 1986) and HAV has recently been assigned to the new genus - hepatovirus (Minor, 1991; Melnick, 1992). Unlike the picornaviruses ECHO 9 (Rifkind et al, 1961) and poliovirus types 1 and 2 (Mayor & Jordan, 1962; Dales et al, 1965), large numbers of virions and paracrystalline arrays could not be found in infected FRhK-4 cells and this may reflect the atypical nature of HAV development. However, inoculation of cultures with high concentration of virus and harvest in five to seven hours, at which time large numbers of poliovirions in crystalline array were found in 50% of cells (Dales et al, 1965) might have produced better results. Alternatively, larger numbers of HAV particles might have been observed if FRhK-4 cells had been incubated at a lower temperature since infectious poliovirus is formed but its release inhibited at 30^{o}C. Poliovirions are not found in cultures incubated at 37^{o}C (Mayor & Jordan, 1962).

The dense cytoplasmic masses of viroplasm characteristic of cells infected with ECHO virus 9 (Rifkind et al, 1961) and poliovirus types 1 and 2 (Mayor & Jordan, 1962; Dales et al, 1965) were not found in HAV-infected FRhK-4 cells. However, picornavirus-like particles were associated with autophagic vacuoles which seemed to be involved in their morphogenesis and it is possible that the matrix of such structures in some way replaces the function of viroplasm.

It is not clear if the co-infection of FRKV in FRhK-4 cultures had any influence on the replication and excretion of HAV. However, Siegl, de Chastonay and Kronauer (1984) showed that more than 90% of hepatitis A antigen remained cell associated and that the small amount of virus recovered in the medium was from detached and degenerated cells. Sonication was found to be a critical factor in extraction of HAV from persistently infected cultures (Simmonds et al, 1985) but, despite the

sonication of detached cells, the number of virions found in FRhK-4 cultures by EM and IEM was disappointingly small. Although EM and IEM are less sensitive than RIA (Kjeldsberg & Siebke, 1985), it is possible that the antigen detected by RIA is in soluble form and not assembled into recognizable virions.

Unlike most of the picornaviruses, HAV establishes persistent rather than cytolytic infections *in vitro*. This restricts production of infectious virus (Siegl et al, 1984) and makes HAV a difficult subject to study (Ticehurst, 1986). Kiernan and co-workers (1987) were unable to recognize HAV in thin sections although the presence of virus and antigen were confirmed by IEM and RIA respectively. However, the morphological features of HAV in thin sections of infected FRhK-4 cells described above have been observed by others. Cell membranes are thought to have an important role in the assembly and release of HAV *in vitro* (Lemon & Binn, 1985): the association of virus with membranes has been demonstrated (Asher, Binn & Marchwicki, 1987; Heinricky et al, 1987; Asher et al, 1988; Tinari et al, 1989) and hepatitis A antigens have been located in membranous inclusions and single membranes by immunoperoxidase staining (Asher et al, 1988). The size of HAV in thin sections has been given as 17 to 20 nm and involvement of autophagic vacuoles has been shown (Asher et al, 1987; 1988). Paracrystalline arrays of hepatitis A virions have not been described (Asher et al, 1987; Heinricky et al, 1987; Asher et al, 1988; Tinari et al, 1989).

The slow rate of virus growth and the minimal effects of most HAV strains on infected cells have hampered the study of HAV replication (Anderson, 1987). Moreover, growth and recovery of HAV from persistently infected FRhK-4 cells has been shown to decrease over two

to three months (Robertson et al, 1988). Fast-growing strains of human HAV with the unusual property of developing a strong CPE, which have recently been developed (Venuti et al, 1985; Nasser & Metcalf, 1987; Tinari et al, 1989), would undoubtedly be more suitable for the study of HAV morphogenesis. However even with a fast-growing strain, solid phase immunoelectron microscopy (SPIEM) of virus concentrated by centrifugation through sucrose appeared to be necessary to find virus readily by negative staining (Venuti et al, 1985).

Neither HAV nor FRKV produced a CPE in FRhK-4 cultures and the presence of the polyomavirus would not have been detected had preparations of culture fluids not been examined by EM. Because adaptation of HAV to growth in cell cultures requires prolonged incubation which would also favour the growth of endogenous or exogenous contaminating viruses, it is important that the identity of strains of HAV should be fully established (Anderson, 1987). The present study indicates that such identification must include EM examination.

4.4. Sizes of papovaviruses

The reported measurements of papovaviruses are given in the Appendix, Table A.3. The sizes show considerable variation, some of which is probably due to the method of preparation, whether virions were isolated or closely packed and whether or not any surrounding membrane was included in the measurement. However, much of the apparent size variation is undoubtedly due to lack of calibration of microscope magnification: in 75 (93%) of the 81 studies (Table A.3) magnification calibration was not mentioned. Although in 43 (53%) of the studies ranges of measurements were given, in only 17 (21%) was the number of virions measured included and in five (6%) was a histogram or graph

shown.

Although it is rarely determined with any degree of accuracy (Table A.3), size has long held a fascination for electron microscopists. In thin sections, there can be no substitute for comparing relative sizes. For example the width of the trilaminar membrane with the diameter of polyomavirions (Figs 30, 31 & 34) and with that of picornavirus-like particles (Figs 35, 36, & 38-40). However, in negatively stained preparations a convenient "omni-present" standard does not exist and the microscopist's memory must be programmed through practice (Madeley & Field, 1988).

Despite this fascination with sizes, there have been numerous reports in which morphologically convincing papovaviruses have been illustrated in the absence of a stated particle diameter including: polyoma (Crawford et al, 1962; Finch, 1974), human wart (Chapman, Drusin & Todd, 1963), RKV (Crawford & Follett, 1967), SV40 (Oshiro et al, 1967), K virus (Jordan & Doughty, 1969; Gleiser & Heck, 1972), BKV (Lecatsas, Prozesky & Scheepers, 1974), HD (Waldeck & Sauer, 1977), LPV (zur Hausen & Gissmann, 1979), FRKV (Parry, Richmond & Gardner, 1983) and BFDV (Dykstra et al, 1984).

Furthermore, a stated diameter of approximately 45 nm, for example in the case of the frog papova-like virus, is unhelpful when thin section images are not quite convincing (Lunger et al, 1965; Lunger, 1966; Granoff et al, 1969) and negatively stained illustrations do not reveal any suggestion of the capsomers characteristic of polyomaviruses (Granoff, 1969; Granoff et al, 1969).

5. GENERAL DISCUSSION

5.1. Discovery of an adventitious polyomavirus, FRKV

The detection of an unexpected virus during work with HAV in continuous lines of fetal rhesus kidney cells has raised the possibility of stocks of viruses grown in these cells being contaminated. This adventitious virus, designated FRKV (fetal rhesus kidney virus), has the fine structural appearance and intranuclear development characteristic of members of the *Papovaviridae*. The morphological features of FRKV, of the various miscellaneous structures observed and of HAV have been considered in Sections 4.1 to 4.3.

Like the members of the *Polyomavirus* genus FRKV grows in cell culture. The mean diameters of 57 nm for human papillomavirus and 47 nm for FRKV are comparable to those described for papillomaviruses and polyomaviruses thus confirming that the virus belongs to the *Polyomavirus* genus. The sizes of FRKV and human papillomavirus determined in the present study have been discussed in relation to those of other papovaviruses in Section 4.4.

5.2. Cell lines in which FRKV grows

Polyomavirions have been detected by EM in uninoculated FRhK-4 cultures obtained from two different sources, Germany and the USA, and in the FRhK-6 cell line. The possibility that these cells had become infected during culture in the Virus Reference Division cannot be discounted. However, these cell lines were among 28 established by Wallace and her colleagues in 1973 (a & b) who at that time reported cellular changes consistent with polyomavirus infection in five fibroblastic and three fetal rhesus kidney cell cultures.

Polyomavirus particles have been observed by EM in culture fluids from uninoculated LLC-MK2, MA104, calf kidney and DBS-FRhL-2 101 cell lines, and FRKV grows in DBS-FRhL-2 103 cultures when these are inoculated with the virus. In addition, polyomavirions have been seen by other workers in uninoculated cell lines. A polyomavirus, not related to SV40, SA12, JCV or BKV, was found in Vero cultures in 1972 by A M Field and S D Gardner (personal communication) and polyomavirions were seen in culture fluids from a bovine kidney cell line, MDBK, by G Burtonboy (personal communication).

Although FRKV grows in primary human embryonic kidney cells, it is not known if the virus will grow in the human diploid cells MRC-5 and WI-38, in which FRhK-adapted HAV is passaged for vaccine production (Provost et al, 1983). However, in a study of ten strains of HAV in Alexander and MRC-5 cells, Siegl, de Chastonay and Kronauer (1984) observed CPE which they ascribed to an FRKV-like agent.

5.3. Significance of EM detection of "naturally" occurring antibody coating

The antibody which coated free FRKV from high speed pellets of FRhK and FRKV-inoculated HEK culture fluids was identical to that surrounding hepatitis A virions after incubation with purified anti-HAV IgG and to antibody molecules illustrated by Almeida and Waterson (1969). Antibody coating was observed when the medium contained fetal or newborn bovine serum, but was not found in preparations of virus from disrupted, washed spontaneously detached cells, nor when serum-free medium was used.

The only possible source of this antibody was the calf serum and these observations were the first indication that FRKV might be bovine.

5.4. Identification of FRKV

5.4.1. IEM demonstrates that STMV, CMKV and LLC-MK2V are strains of FRKV

Immunoelectron microscopy has been effectively used to identify many strains of polyomaviruses (Penney et al, 1972; Albert & ZuRhein, 1974; Field et al, 1974; Reissig et al, 1976; Tumilowicz et al, 1979) and I have applied this technique to the study of FRKV.

The IEM results clearly demonstrate that the polyomaviruses isolated from FRhK-4 and FRhK-6 cultures, FRK4V and FRK6V, are indistinguishable from STMV and CMKV (Table 3.1) and are not related to SV40, SA12, JCV or BKV (Table 3.2). These findings are supported by immunofluorescence studies (S D Gardner). In addition, FRKV has biological properties similar to STMV and CMKV: cytoplasmic vacuolation of HEK and CK cells is a prominent feature (S D Gardner and J V Parry) and FRKV, like STMV, fails to agglutinate erythrocytes of various animal species.

IEM has also shown that FRKV is antigenically closely related to the LLC-MK2 polyomavirus (Table 3.3) and, using purified goat anti-bovine IgG, has confirmed that the antibody which coats free virus in culture fluids is bovine. The MA104 polyomavirus is also related to FRKV (Section 3.20).

It was concluded, therefore, that the polyomaviruses STMV and thus HD, CMKV, LLC-MK2V and MA104V, all of which have independently been isolated from monkey kidney cell lines in different laboratories, are strains of the same virus, FRKV.

5.4.2. IEM evidence that FRKV and FRKV-like viruses are bovine

That FRKV is a bovine virus has also been clearly demonstrated by IEM. WRSV was the first recognized bovine polyomavirus (Coackley et al, 1980) and the International Committee on Taxonomy of Viruses has proposed that it be called *Polyomavirus bovis* (Frisque, 1991). FRKV is indistinguishable by IEM from WRSV (Table 3.1). Furthermore, IEM has shown that FRKV is closely related to CK29/2V and CK10V, two of the polyomaviruses more recently isolated in calf kidney cultures (Tables 3.6 & 3.7).

IEM has also demonstrated that there are large or moderate amounts of antibody against FRK4V, FRK6V and WRSV in several newborn and fetal bovine serum pools (Table 3.1). The presence of bovine antibody against FRK4V has been confirmed by EM examination of CIE precipitin lines formed with purified preparations of IgG, RDE-treated samples and a number of sera from individual animals. Further confirmation was provided by additional CIE studies (J V Parry) in which anti-FRKV was not detected in sera from rhesus monkeys but was found in 48% of cattle sera. Moreover, FRKV adapted readily to growth in secondary calf kidney cultures (J V Parry and S D Gardner) and grew more rapidly and to higher titres than in FRhK-4 cells.

The amounts of antibodies against FRKV and WRSV in pools of newborn bovine sera are identical to those in the antisera specifically raised against STMV and CMKV (Table 3.1). It was concluded that STMV, HD, FRKV, CMKV and LLC-MK2V are not latent simian viruses but that these antigenically similar viruses are all bovine and are strains of WRSV. It is probable that the various monkey kidney cell lines have become infected through the use of contaminated batches of bovine serum in the

culture media.

5.4.3. Additional evidence that STMV and CMKV are bovine viruses

Evidence of natural infection with many of the polyomaviruses has been confirmed by the demonstration of specific antibody in the host species (Table 5.1). STMV is unusual in that antibody against the virus has not been found in stump-tailed macaques (Table 5.1). To account for this it has been proposed that, following congenital transmission (Shah et al, 1977), the virus remains unexpressed (Shah et al, 1975). However, this is unlikely because stump-tailed macaques produce antibody when inoculated with the virus (Shah et al, 1975). A more plausible explanation for the absence of naturally occurring antibody is that STMV is bovine in origin (Table 3.1). Furthermore, STMV was often aggregated and, despite Genetron-treatment, the remaining "amorphous substance" between the virions in clumps illustrated by Reissig and her colleagues (1976) strongly resembles antibody.

CMKV had initially been identified as a strain of STMV but, following preliminary reports suggesting that FRKV and STMV were bovine and that bovine sera contained anti-FRKV (Richmond et al, 1983; Parry et al, 1983), its origin was further investigated (Wognum et al, 1984). Anti-CMKV could not be found in sera from cynomolgus macaques, but was detected by IF in 44% of 57 cattle sera, four newborn calf serum pools and six of 26 bovine colostrum samples. Wognum and his colleagues (1984) concluded that CMKV, like FRKV, was a bovine virus and that contaminated serum used in the culture medium might have been the source of infection. The complete nucleotide sequence of CMKV was described by Schuurman, Sol and van der Noorda (1990) and the virus renamed bovine polyomavirus.

Table 5.1. Presence of naturally occurring antibody in the reported hosts of polyomaviruses

Virus	Reported host		Reference
K virus	mouse	ab +	Holt, 1959
polyoma	mouse	ab +	Rowe et al, 1958
SV40	rhesus monkey	ab +	Sweet & Hilleman, 1960a
SA12	chacma baboon	ab +	Valis et al, 1977
RKV	rabbit	ab +	Hartley & Rowe, 1964
JCV	man	ab +	Padgett & Walker, 1973
SV40-PML	man	ab +	Weiner et al, 1972; Penney et al, 1972
BKV	man	ab +	Gardner, 1973; Coleman, Gardner & Field, 1973
SPV	pig	ab +	Newman & Smith, 1972
STMV	stump-tailed macaque	ab -	Shah et al, 1975
LPV	African green monkey	ab +	zur Hausen & Gissmann, 1979
LPV	man	ab +	Brade, Muller-Lantzsch & zur Hausen, 1980
WRSV	calf	ab +	Table 3.1
FRKV	calf	ab +	Parry, Richmond & Gardner, 1983
CMKV	calf	ab +	Wognum et al, 1984
CKV	calf	ab +	Westcott et al, 1987
PP-2	baboon	ab +	Gardner et al, 1989

ab + = antibody detected; ab - = antibody not detected

It is not known if there is naturally occurring host antibody against the following: HPV and LHV - hamster, OMKV - owl monkey, FPV - frog, BFDV - budgerigar or RPV - rat

5.5. Intrauterine infection in cattle and presence of viral antibody

5.5.1. Isolation of viruses

Many viruses are known to cause intrauterine infection in cattle including bovine adenovirus types 1, 4, 6 and 8 (Bartha & Mate, 1983), bovine diarrhoea virus (Hassan & Scott, 1986), bovine leukaemia virus (Kono et al, 1983) and bovine syncytial virus (Van Der Maaten et al, 1973). These viruses were isolated from tissues of fetal or colostrum-deprived calves and infection was naturally acquired *in utero* from the mother. The longer that gestation progressed, the less likely it was that the virus crossed the placenta, particularly if the virus was of low pathogenicity (Dunne et al, 1973).

Since viruses have been isolated from fetal and newborn calves, it is not surprising that there have been isolations of several viruses from commercial pools of fetal bovine sera. Such viruses include bovine diarrhoea virus (Fedoroff et al, 1972; Molander et al, 1972; Kniazeff et al, 1975), bovine enteroviruses (Fedoroff et al, 1972; Kniazeff et al, 1975), bovine herpesviruses (Fedoroff et al, 1972; Molander et al, 1972; Kniazeff et al, 1975), parainfluenzavirus type 3 (Fedoroff et al, 1972; Kniazeff et al, 1975), reovirus type 1 (Karpas, Cawley & Nagington, 1977) and uncharacterized viruses (Fedoroff et al, 1972; Kniazeff et al, 1975).

5.5.2. Presence of viral antibody in bovine fetuses and newborn calves

In cattle, transmission of immunity occurs after birth. Instead of maternal antibody being transferred across the placenta to the fetus as in man, newborn calves acquire antibody by ingesting colostrum (Brambell, 1958). The presence of specific antibody in the fetus and in

pre-colostral calves is regarded as good evidence of fetal infection (Dunne et al, 1973; Sato et al, 1980) and has been demonstrated for several viruses (Table 5.2).

Antibody-producing cells in aborted fetuses (Dunne et al, 1973) and high concentration of IgM antibodies in fetal fluids (Schultz, Confer & Dunne, 1971; Dunne et al, 1973) provide further evidence of fetal infection. Bovine fetuses, like adults, are capable of recovery from viral infections with little lasting effect (Dunne et al, 1973). In addition, antibodies against bovine diarrhoea virus (Calafat, Hageman & Ressang, 1976), bovine parvovirus type 1 (Storz et al, 1972) and bovine rotavirus (Offit et al, 1984) have been detected in pools of fetal bovine serum.

The presence of antibody against FRKV-like polyomaviruses in some fetal bovine sera (Tables 3.1, 3.3 & 3.6) indicates that infection of the fetus has occurred *in utero* and that batches of serum could contain infectious virus. Furthermore, since many polyomaviruses have been found in kidney or kidney cell cultures (Table 1.1), fetal bovine kidney might also contain polyomaviruses.

5.6. Isolation of polyomaviruses in calf kidney cultures exposed to fetal bovine serum that contained infectious virus and antibody

Polyomavirions were found by EM in all but two of the 23 batches of CK cells for which culture fluid included fetal bovine serum U720201D either continuously (Section 3.21) or for short periods (Table 3.5). In many harvests virus particles were densely coated with antibody (Tables 3.4 & 3.5). Polyomavirions could not be found in any of the cultures for which gamma-irradiated serum had been used nor in the suspect serum.

Table 5.2. Viral antibodies detected in bovine colostrum, fetal and pre-colostral calf sera

Virus	Source of antibody	Reference
bovine adenovirus type 2	fetal & pre-colostral bovine sera	Sato et al, 1980
types 4, 6 & 8	pre-colostral bovine sera	Bartha & Mate, 1983
bovine coronavirus	fetal & pre-colostral bovine sera	Sato et al, 1980
bovine diarrhoea virus	fetal bovine sera	Kniazeff, Rimer & Gaeta, 1967
	sera from pre-colostral calves	Kendrick, 1971
	sera from aborted fetuses	Dunne et al, 1973
	14% of fetal bovine sera	Bolin et al, 1991
bovine enteroviruses, various types	41% of sera from aborted fetuses	Dunne et al, 1973
bovine leukaemia virus	fetal bovine serum	Kono et al, 1983
parainfluenzavirus type 3	53% of sera from aborted fetuses	Dunne et al, 1973
bovine parvovirus	fetal & pre-colostral bovine sera	Sato et al, 1980
type 1	fetal bovine sera	Storz et al, 1972
polyomavirus FRKV	colostrum-deprived calves & colostrum	Parry et al, 1983b
CMKV	colostrum	Wognum et al, 1984
infectious bovine rhinotracheitis virus	sera from aborted fetuses	Dunne et al, 1973
bovine rotavirus	fetal & pre-colostral bovine sera	Sato et al, 1980
	sera of cesarian-derived calves	Offit et al, 1984
	pre-colostral bovine sera	Offit et al, 1984

It is clear that the polyomaviruses from CK31/1 and CK33/1 originated from the serum since virus could not be found in the control cultures, CK31/2 and CK33/2, which had been in contact only with gamma-irradiated serum. The detection of FRKV antigen by RIA (J V Parry) in a harvest from one of the 13 cultures exposed only to gamma-irradiated serum presumably represents virus which originated from the calf kidney.

It is probable that CK29/1V and CK29/2V were also derived from the serum but the origin of the 18 polyomaviruses from CK8 to CK28 cultures is less clear. It is reasonable to suppose that many of these isolates were exogenous having come from the serum but, since CKV has been isolated from kidney cultures from 28% of clinically normal calves (Westcott et al, 1987), at least some of these viruses are likely to have been endogenous.

Both CK29/2V, from cultures on medium containing equine serum, and CK10V (Tables 3.6 & 3.7a) were found by IEM to be very similar to FRK4V, FRK6V and WRSV. The increased numbers of CK8V, CK9V and CK12V particles observed (Table 3.7b) may account for the results with the anti-STMV sera, but does not explain the abnormal reactions between these viruses and both of the newborn bovine sera, which reacted strongly in IEM with all other FRKV-like viruses (Tables 3.1, 3.3 & 3.6). These three CK isolates appear to be related but not identical to FRKV and may have been derived from the calf kidney cells. The relationships between the various calf kidney isolates, and whether they originate from the kidney or the serum in the culture medium, might be clarified by further investigation, for example using PCR.

That virus particles could not be found in the suspect serum U720201D may simply reflect the relative insensitivity of EM. Alternatively, it

is possible that complete virions were not present but that the serum contained infectious DNA. Chambers, Hsia and Ito (1966) have shown that inoculation of rabbit kidney cells with DNA extracted from RKV (Ito, Hsia & Evans, 1966) results in characteristic CPE and that virus particles can be observed by negative stain and thin section EM.

It was concluded that fetal bovine serum U720201D contained both antibody and infectious virus (or possibly DNA). In addition, antibody against the LLC-MK2V was present in the fetal bovine serum that was implicated in the contamination of both the LLC-MK2 and MA104 cell lines (Section 3.20). Since antibody has been found by IEM in 75% of other sera supplied for cell culture (Table 3.1), it is probable that some of these pools are also infectious. Although the presence of antibody indicates that the serum might be infectious, the absence of antibody cannot be taken to imply that the serum does not contain virus.

Further evidence of the potential infectiousness of serum which contains antibody has been provided by Westcott and colleagues (1987) who found anti-CKV in sera from 17 (52%) of 33 calves, including 14 of the 18 calves from the kidneys of which virus was isolated. More recently, Schuurman and his co-workers (1991b) have amplified bovine polyomavirus (CMKV) DNA in 70% of 20 commercial batches of calf sera. The presence of DNA correlated well with virus isolation: virus was isolated from five DNA-containing sera, but not from five sera that were DNA-negative. Of 11 serum samples in which antibody was found, bovine polyomavirus (CMKV) DNA was detected in seven (64%) (Schuurman et al, 1991b).

5.7. The sensitivity of EM and the use of antibody-coated grids

EM is not a sensitive technique; it is generally accepted that large numbers of virions, approximately 10^6 particles per ml, are necessary for detection (Galasso, 1967; Mathews & Buthala, 1970; Monroe & Brandt, 1970; Flewett & Boxall, 1976).

It is not surprising, therefore, that FRKV antigen should have been detected by RIA in two cultures in which virus particles had not been seen. It is, perhaps, more surprising that polyomavirions were found in four CK cultures which were negative by RIA. In two of these instances the dense antibody surrounding the virions might explain the RIA-negative results. It is not clear why RIA failed to detect FRKV in the remaining two EM-positive cultures, but this may also be due to the specificity of RIA. These observations demonstrate the importance of EM as a "catch-all" method for detecting viruses.

The variation in numbers of virions found in different preparations of the same culture fluid and on repeat examination of the grids (Table 3.8) clearly illustrates the problems facing electron microscopists when virus concentrations are low. The use of antibody-coated grids did not improve the sensitivity of EM but it is possible that pre-coating the grids with staphylococcal protein A might have been more successful (Shukla & Gough, 1979; Katz & Kohn, 1984; Kjeldsberg & Siebke, 1985; Humphrey et al, 1988).

5.8. Implications of the discovery of FRKV

5.8.1. Cell lines currently used in vaccine production

Both FRhK-4 and FRhK-6 were among 27 of the cell lines that Wallace and her colleagues (1973b) rejected as candidates for virus vaccine production. Although CPE reminiscent of infection with SV40 developed in some cultures, attempts to demonstrate viral contaminants were unsuccessful (Wallace et al, 1973b). The DBS-FRhL-2 cell line, however, met the requirements for virus vaccine substrates despite the presence in some cultures of vacuolated cells which became detached from the flasks (Wallace et al, 1973a). Repeated tests on DBS-FRhL-2 cells for viral agents were also negative but in neither of these studies (Wallace et al, 1973 a & b) did tests for adventitious viruses include EM.

An experimental live HAV vaccine, using strain CR326F attenuated by several passages in FRhK-6 cultures followed by MRC-5 cells, has been tested in adult human volunteers most of whom developed anti-HAV (Provost et al, 1986). In a dose-escalating study this attenuated HAV vaccine was well tolerated and highly immunogenic (Midthun et al, 1991). Seroconversion occurred in recipients of another experimental live HAV vaccine developed from strain H2, which was isolated and passaged in newborn monkey kidney cells then passed in human lung diploid cells (Mao et al, 1989).

A live oral rotavirus vaccine has been developed from calf diarrhoea virus, obtained after 140 passages in primary fetal bovine kidney cells (Delem, Lobmann & Zygraich, 1984). For the trial in adults virus was produced in primary fetal bovine kidney cells (Vesikari et al, 1983). Since bovine fetuses free from bovine diarrhoea virus are difficult to obtain, *Cercopithecus* monkey kidney cells were subsequently used to

prepare experimental batches of vaccine (Delem et al, 1984). Side effects were not observed in infants aged eight to 11 months who received this live oral rotavirus vaccine and were followed up serologically and clinically during a rotavirus subgroup 2 epidemic (Vesikari et al, 1984).

Two vaccines have been prepared using the DBS-FRhL-2 cell line. A live dengue 4 vaccine has been attenuated by passage in primary dog kidney cells followed by DBS-FRhL-2 cultures and tested in yellow fever-immune volunteers (Eckels et al, 1984). A rabies vaccine has been prepared in DBS-FRhL-2 cells and the virus inactivated using B-propiolactone (Burgoyne et al, 1985).

The present study has shown that FRKV is capable of growing in several cell lines that are currently being used in vaccine production including FRhK-6, DBS-FRhL-2, monkey and bovine kidney. Although, in a study in 1984, Provost and his co-workers did not find evidence of polyomavirus in cultures inoculated with HAV passaged in FRhK-6 cells, it is unclear whether there has been continued monitoring of vaccine strains of HAV or whether the cell lines used in the production of other vaccines have even been examined for contamination with FRKV.

5.8.2. Transformation and tumour induction by FRKV-like viruses

HD virus is known to transform Vero cultures (Waldeck & Sauer, 1977) and the early gene products of bovine polyomavirus (CMKV) are capable of transforming murine cells *in vitro* (Schuurman et al, 1992). After injection of immunocompromized rats with CMKV-transformed murine cells, primary fibrosarcomas infiltrating neighbouring muscles were observed at the site of inoculation. Metastases were found in the peripheral lymph

nodes of some animals (Schuurman et al, 1992).

5.8.3. FRKV in man

Natural transmission of SV40 from rhesus monkeys to man has been reported (Shah, 1966) and antibody against SV40 was detected in 27% of monkey handlers (Shah, 1972). FRKV grows readily in HEK cells *in vitro* and it appears that the virus can also infect man. Anti-FRKV has been detected in a high percentage of people who come in close contact with cattle, for example in 70% of veterinary surgeons, 50% of cattle farmers and 40% of abbatoir workers compared with 0.5% of 256 blood donors (Parry & Gardner, 1986). Further study should be undertaken to confirm that active infection does occur in man and if there are any harmful effects.

5.9. Prevention of contamination with FRKV

"Serum is the most unstandardized, variable, and unpredictable of the ingredients that go into tissue culture media" (Fedoroff et al, 1972) and it has been suggested that as long as serum is necessary for cell culture propagation, the threat of virus contamination will be present (Kniazeff et al, 1975). A WHO review of tests on virus vaccines recommended that serum should be from identified animals, gamma-irradiation should be used and tests for bovine viruses should be included (Anon, 1982); irradiation of serum at -40^{0}C has been suggested (Erickson et al, 1989).

However, the use of gamma-irradiated serum is not without problems. Gamma-irradiation did not completely eliminate virus from a batch of serum contaminated with high titres of CMKV (bovine polyomavirus) though it resulted in a ten-fold reduction in infectivity (B van Steenis,

unpublished results, Schuurman, van Steenis & Sol, 1991). Furthermore, although irradiated serum has proved satisfactory for cell culture in the present study, others have found that the growth-stimulating properties of the serum were severely reduced by the irradiation process (Schuurman et al, 1991b).

The finding of anti-FRKV in only 0.5% of 219 vaccinees (Parry & Gardner, 1986) indicates that there is little risk of infection from undetected FRKV contamination of current cell culture-derived vaccines, but wider use of primate kidney cells as substrates for new vaccines may increase this risk. The most effective method to prevent contamination with FRKV would be to avoid the use of calf serum, but cell cultures do not grow well when maintained for long periods in serum-free medium or medium containing equine serum (S D Gardner, personal communication). The use of serum from identified animals would reduce the risk of contamination since commercial batches of serum usually consist of the pooled sera collected from more than 100 calves.

The use of fetal or newborn bovine sera for cell culture is widespread and all users, not just manufacturers of vaccines and other biological reagents, should be aware of the potential hazards associated with FRKV. To reduce the risk of contamination, calf serum batches should be screened by highly sensitive methods before use for cell culture and PCR appears to provide a sound basis for predicting the presence of infectious virus in serum batches (Schuurman et al, 1991b). Furthermore, high speed pellets of cell culture fluids should be examined by EM since FRKV infection is frequently non-cytopathic and therefore likely to be unrecognized unless attempts to detect it are made.

APPENDIX

Papovavirus morphology: summary of the literature

Table A.1a. Morphological features described or illustrated in thin sections of papovaviruses

Virus	in	ic	sph	cry	fil	mem	Specimen	Reference
Shope	in	-	sph	-	-	-	rabbit papilloma	Haguenau et al, 1960
	-	-	sph	-	-	-	rabbit papilloma, purified virus	Haguenau et al, 1960
human	in	ic	sph	cry	-	-	human papilloma	Bunting, 1953
papilloma	in	-	sph	cry	-	-	human papilloma	Williams, Howatson & Almeida, 1961
	in	ic	sph	cry*	-	-	human papilloma	Chapman et al, 1963
	in	-	sph	cry	-	-	human papilloma	Howatson, 1973
K virus	in	ic	sph	-	-	-	mouse lung	Dalton et al, 1959
	in	ic	sph	cry	fil	mem	mouse lung	Dalton, Kilham & Zeigel, 1963
	in	ic	sph	cry	-	mem	mouse lung, liver & slpeen	Jordan & Doughty, 1969
	in	-	sph	-	-	-	mouse liver & spleen	Gleiser & Heck, 1972
	in	-	sph	cry	-	-	mouse embryo cell culture	Greenlee, Dodd & Oster-Granite, 1982
polyoma	in	ic	sph	cry	-	mem	mouse cell culture	Banfield, Dawe & Brindley, 1959
	in	ic	sph	cry	fil	-	mouse embryo cell culture	Bernhard, Febvre & Cramer, 1959
	in	ic	sph	-	-	mem	mouse embryo cell culture	Negroni et al, 1959
	in	ic	sph	cry	fil	mem*	hamster kidney	Howatson & Almeida, 1960a
	in	ic	sph	cry	fil	mem*	hamster kidney	Howatson & Almeida, 1960b
	in	ic	sph	cry	-	mem	hamster kidney & various cultures	Howatson et al, 1960
	in	ic	sph	cry	fil	mem	hamster kidney cell culture	Howatson, 1962
	in	-	sph	cry	fil	mem	mouse fibroblast culture	Dalton et al, 1963
	in	ic	sph	cry	fil	mem	mouse embryo cell culture	Mattern, Takemoto & Daniel, 1966
	in	ic	sph	-	fil*	mem	hamster kidney	Howatson, 1973

continued ...

For table abbreviations, please see A.1d

Table A.1b. Morphological features described or illustrated in thin sections of papovaviruses

Virus	in	ic	sph	cry	fil	mem	Specimen	Reference
polyoma	in	ic	sph	cry	fil	mem	mouse spinal cord	Sebesteny et al, 1980
(contd)	in	-	sph	cry*	fil	mem	Friend leukaemic cell culture	Taddei et al, 1982
	in	ic	sph	cry	-	-	mouse spinal cord	McCance et al, 1983
SV40	in	ic	sph	-	-	mem	human & monkey kidney cell culture	Gaylord & Hsiung, 1961
	in	ic	sph	cry	-	mem	monkey kidney cell culture	Mayor et al, 1962
	in	ic	sph	cry	-	mem*	monkey kidney cell culture	Granboulan et al, 1963
	in	ic	sph	cry	-	mem	human foreskin cell culture	Petursson et al, 1966
	in	ic	sph	cry*	-	mem*	monkey kidney cell culture	Oshiro et al, 1967
	in	ic	sph	-	-	mem*	monkey kidney cell culture	Howatson, 1973
	in	ic	sph	cry	-	-	monkey lung & kidney lesion	Sheffield et al, 1980
SA12	in	ic	sph	-	-	mem	monkey kidney cell culture	Malherbe & Strickland-Cholmley, 1973
	in	ic	sph	-	-	mem*	monkey kidney cell culture	Lecatsas, Malherbe & Strickland-Cholmley, 1977
RKV	in	ic	sph	-	fil	mem	rabbit kidney cell culture	Chambers et al, 1966
PML	in	ic	sph	cry	fil	mem	human brain	Howatson et al, 1965
	in	ic	sph	-	fil	-	human brain	Silverman & Rubinstein, 1965
	in	-	sph	cry	fil	mem	human brain	ZuRhein & Chou, 1965
	in	ic	sph	cry*	fil*	mem	human brain	ZuRhein, 1969
JCV	in	ic	sph	cry	fil	mem	human brain & cell culture	Padgett et al, 1971

continued ...

Table A.1c. Morphological features described or illustrated in thin sections of papovaviruses

Virus	in	ic	sph	cry	fil	mem	Specimen	Reference
SV40-PML	in	ic	sph	-	fil	mem	human brain & monkey cell culture	Weiner et al, 1972
JCV-COL	in	ic	sph	cry	fil	mem	human brain & cell culture	Field et al, 1974
FPV	in	-	sph	cry	-	-	frog kidney	Lunger et al, 1965
	in	-	sph	cry	-	-	frog kidney	Lunger, 1966
	in	ic	sph	-	-	-	frog kidney	Granoff et al, 1969
HPV	in	ic	sph	cry	fil	-	hamster skin tumour	Graffi et al, 1968
LHV	in	ic	sph	-	fil	mem	hamster kidney, spleen, brain etc	Hannoun et al, 1974
BKV	in	ic	sph	cry	-	-	human urine, ureter & cell culture	Gardner et al, 1971
	in	ic	sph	cry	-	mem	human fetal cell culture	Lecatsas et al, 1974
SPV	in	-	sph	-	fil	-	pig kidney cell culture	Tumilowicz et al, 1979
OMKV	in	-	sph	-	-	-	owl monkey kidney cell culture	King et al, 1972
STMV	in	ic	sph	-	-	mem*	macaque kidney cell culture	Rangan et al, 1974

continued ...

Table A.1d. Morphological features described or illustrated in thin sections of papovaviruses

Virus	in	ic	sph	cry	fil	mem	Specimen	Reference
M-PML	in	ic	sph	cry	-	-	monkey brain, PML-like lesion	Gribble et al, 1975
HD	-	ic	sph	-	-	mem	monkey kidney cell culture	Waldeck & Sauer, 1977
LPV	in	-	sph	-	-	-	monkey lymphoblastoid cell culture	zur Hausen & Gissmann, 1979
WRSV	in	ic	sph	cry	fil	-	calf kidney cell culture	Coackley et al, 1980
BFDV	in	-	sph	-	fil	-	budgerigar heart, kidney etc	Bernier et al, 1981
	in	-	sph	-	-	-	chicken fibroblast culture	Bozeman et al, 1981
	in	-	sph	-	-	-	budgerigar kidney	Davis et al, 1981
	in	-	sph	-	fil	-	budgerigar kidney & cell culture	Dykstra & Bozeman, 1982
	in	-	sph	-	-	-	various birds liver & spleen	Jacobson et al, 1984
FRKV	in	ic	sph	cry	fil	mem*	fetal rhesus kidney cell culture	Richmond et al, 1984
RPV	in	ic	sph	cry	-	-	rat parotid gland	Ward et al, 1984

in = intranuclear; ic = intracytoplasmic; sph = spherical particles; cry = paracrystalline formation; fil = filamentous particles; mem = particles associated with membranes; - = not present or not described; * particularly pronounced

Table A.2a. Morphological features described or illustrated in air dried, metal shadowed, negatively and positively stained preparations of papovaviruses

Virus	Prep	sph	fil	min	abr	mem	cap	a/s	SRP	Specimen	Reference
Shope	AD	sph	-	-	-	-	-	-	-	rabbit papilloma	Sharp et al, 1942
	SP	sph	-	-	-	-	-	-	-	rabbit papilloma	Sharp et al, 1946
	NS	sph	fil	-	-	-	-	-	-	rabbit papilloma	Williams, Kass & Knight, 1960
	AD	sph	-	-	-	-	-	-	-	rabbit papilloma	Williams et al, 1960
	SP	sph	fil	-	abr	-	cap*	a/s	-	rabbit papilloma	Breedis et al, 1962
	NS	sph	fil	-	-	-	cap	-	-	rabbit papilloma	Howatson & Crawford, 1963
	NS	sph	fil*	min*	-	-	cap*	-	-	rabbit papilloma	Finch & Klug, 1965
	NS	sph	-	-	-	-	-	-	-	rabbit papilloma	Howatson, 1973
human papilloma	SP	sph	-	-	-	-	-	-	-	human papilloma	Bunting, 1953
	SP	sph	-	-	-	-	-	-	-	human papilloma	Williams et al, 1961
	NS	sph	fil	-	abr	-	-	-	-	human papilloma	Howatson, 1962
	NS	sph	fil*	-	abr	-	cap	-	-	human papilloma	Noyes, 1964
	NS	sph	-	-	-	mem	cap	-	-	human papilloma	Klug & Finch, 1965
	SP	sph	-	-	-	-	cap	-	-	human papilloma	Smith et al, 1965
	PS	sph	-	-	-	-	cap	-	-	human papilloma	Smith et al, 1965
	NS	sph	-	-	-	-	cap	-	-	human papilloma	Smith et al, 1965
	NS	sph	-	-	-	-	-	a/s	-	human papilloma	Almeida, Oriel & Stannard, 1969
	NS	sph	fil	-	abr	-	-	-	-	human papilloma	Howatson, 1973

continued ...

For table abbreviations, please see A.2e

Table A.2b. Morphological features described or illustrated in air dried, metal shadowed, negatively and positively stained preparations of papovaviruses

Virus	Prep	sph	fil	min	abr	mem	cap	a/s	SRP	Specimen	Reference
K virus	NS	sph	-	-	-	-	-	a/s	-	mouse lung	Mattern et al, 1963
	NS	sph	-	-	-	mem	-	a/s	-	mouse kidney	Parsons, 1963
polyoma	NS	sph	-	-	-	mem	-	a/s	-	mouse embryo cell culture	Wildy et al, 1960
	NS	sph	fil	-	-	-	-	-	-	mouse embryo cell culture	Smith & Benyesh-Melnick, 1961
	NS	sph	-	-	abr	-	-	-	SRP	mouse fibroblast culture	Crawford et al, 1962
	NS	sph	fil	-	-	mem	-	a/s	SRP	hamster kidney cell culture	Howatson, 1962
	NS	sph	fil	-	abr	-	-	-	-	mouse embryo cell culture	Almeida, Cinader & Howatson, 1963
	NS	sph	-	-	-	mem	-	a/s	-	mouse fibroblast culture	Crawford & Crawford, 1963
	NS	sph	-	-	-	-	cap	-	-	mouse embryo cell culture	Howatson & Crawford, 1963
	NS	sph	-	-	-	-	-	-	SRP	-	Howatson et al, 1965
	NS	sph	fil*	min*	-	mem*	cap*	a/s	-	mouse embryo cell culture	Mattern et al, 1967
	NS	sph	fil	-	abr	-	-	-	-	-	Almeida & Waterson, 1969
	NS	sph	fil	-	-	-	-	a/s	SRP	mouse fibroblast culture	Lecatsas & Mallucci, 1972
	NS	sph	fil	-	-	-	-	-	-	hamster kidney	Howatson, 1973
	NS	sph	-	-	-	-	-	-	-	-	Finch, 1974
	NS	sph	-	-	-	-	-	-	-	mouse spinal cord	Sebesteny et al, 1980
SV40	NS	sph	fil	min	-	mem	-	-	-	monkey kidney cell culture	Bernhard, Vasquez & Tournier, 1962
	NS	sph	-	-	-	mem	-	a/s	-	monkey kidney cell culture	Mayor, Jamison & Jordan, 1963
	NS	sph	-	-	-	mem*	-	a/s*	SRP	monkey kidney cell culture	Black et al, 1964
	NS	sph	-	min*	-	-	-	-	-	monkey kidney cell culture	Anderer et al, 1967
	NS	sph	-	min*	abr	-	cap	-	SRP	monkey kidney cell culture	Koch et al, 1967

continued ...

Table A.2c. Morphological features described or illustrated in air dried, metal shadowed, negatively and positively stained preparations of papovaviruses

Virus	Prep	sph	fil	min	abr	mem	cap	a/s	SRP	Specimen	Reference
SV40	SP	sph	-	-	-	-	-	-	-	monkey kidney cell culture	Koch et al, 1967
(contd)	NS	sph	-	min	-	mem	-	-	-	monkey kidney cell culture	Howatson, 1973
SA12	NS	sph	-	-	-	-	-	a/s	SRP	monkey kidney cell culture	Lecatsas et al, 1977
	NS	sph	-	-	-	-	-	a/s	-	monkey kidney cell culture	Valis et al, 1977
RKV	NS	sph	fil	-	-	mem*	-	a/s	SRP	rabbit kidney cell culture	Chambers et al, 1966
	NS	sph	fil	-	-	-	-	-	SRP	rabbit kidney cell culture	Crawford & Follett, 1967
PML	NS	sph	-	-	-	mem	-	a/s	-	human brain	Howatson et al, 1965
	NS	sph	fil	-	-	-	cap	-	-	human brain	Schwerdt et al, 1966
	NS	sph	-	-	-	mem	-	a/s	-	human brain & cell culture	Padgett et al, 1971
	NS	sph	-	-	-	-	-	a/s	-	human brain	Howatson, 1973
JCV	NS	sph	fil	min	-	mem	-	a/s	-	human brain & glial cell culture	Albert & ZuRhein, 1974
SV40-PML	NS	sph	-	-	-	mem	-	a/s	-	human brain & monkey cell culture	Weiner et al, 1972
	NS	sph	-	min	-	-	-	-	SRP	human brain & glial cell culture	Albert & ZuRhein, 1974
JCV-COL	NS	sph	-	-	-	-	-	-	-	human brain & cell culture	Field et al, 1974
FPV	NS	sph	-	-	-	mem	-	a/s	-	frog embryo cell culture	Granoff et al, 1969
	NS	sph	-	-	-	mem	-	a/s	-	frog embryo cell culture	Granoff, 1969

continued ...

Table A.2d. Morphological features described or illustrated in air dried, metal shadowed, negatively and positively stained preparations of papovaviruses

Virus	Prep	sph	fil	min	abr	mem	cap	a/s	SRP	Specimen	Reference
HPV	NS	sph	-	-	-	-	-	-	-	hamster skin tumour	Graffi et al, 1968
BKV	NS	sph	-	-	-	-	-	-	-	human urine, ureter & cell culture	Gardner et al, 1971
	NS	sph	fil	-	-	-	-	a/s	SRP	monkey kidney cell culture	Albert & ZuRhein, 1974
	NS	sph	-	-	-	mem*	-	-	-	human fetal cell culture	Lecatsas et al, 1974
	NS	sph	fil	min	-	mem	cap	-	-	human urine & cell culture	Lecatsas & Prozesky, 1975
SPV	NS	sph	fil	-	-	mem*	-	a/s*	-	pig kidney cell culture	Newman & Smith, 1972
	NS	sph	-	-	-	mem*	-	-	SRP*	pig kidney cell culture	Tischer et al, 1974
	NS	sph	-	-	-	mem	-	a/s	SRP*	pig kidney cell culture	Tumilowicz et al, 1979
STMV	NS	sph	-	-	-	mem*	-	a/s*	SRP	monkey kidney cell culture	Reissig et al, 1976
HD	NS	sph	-	-	-	-	-	a/s	-	monkey kidney cell culture	Waldeck & Sauer, 1977
LPV	NS	sph	-	-	-	mem	-	a/s	-	monkey lymphoblastoid cell culture	zur Hausen & Gissmann, 1979
	NS	sph	-	-	-	mem	-	a/s	-	human lymphoblastoid cell culture	Brade et al, 1980

continued ...

Table A.2e. Morphological features described or illustrated in air dried, metal shadowed, negatively and positively stained preparations of papovaviruses

Virus	Prep	sph	fil	min	abr	mem	cap	a/s	SRP	Specimen	Reference
WRSV	NS	sph	-	-	-	mem*	-	a/s	SRP	calf kidney cell culture	Coackley et al, 1980
BFDV	NS	sph	-	-	-	-	-	a/s	-	skin homogenate	Bernier et al, 1981
	NS	sph	fil	-	-	mem	-	a/s	-	budgerigar kidney & cell culture	Dykstra & Bozeman, 1982
	NS	sph	-	-	-	mem	-	a/s	-	chicken fibroblast culture	Dykstra et al, 1984
	NS	sph	-	-	-	-	-	-	-	chicken embryo cell culture	Muller & Nitschke, 1986
FRKV	NS	sph	-	-	-	-	-	-	-	fetal rhesus kidney cell culture	Richmond et al, 1983
	NS	sph	-	-	-	-	-	a/s	SRP	fetal rhesus kidney cell culture	Parry et al, 1983
	NS	sph	fil	min	-	mem*	-	a/s*	SRP	fetal rhesus kidney cell culture	Richmond et al, 1984
CMKV	NS	sph	-	-	-	-	-	-	-	cynomolgus macaque kidney cell culture	Wognum et al, 1984
CKV	NS	sph	fil	-	-	mem*	-	-	SRP	calf kidney cell culture	Westcott et al, 1987
PP-2	NS	sph	-	-	-	-	-	-	-	baboon kidney cell culture	Gardner et al, 1989

Prep = preparation: AD = air dried; SP = metal shadow preparation; NS = negative stain; PS = positive stain

sph = spherical particles; fil = filamentous particles; min = "mini" or "mini mini" papovas; abr = aberrant particles; mem = particles associated with membranes; cap = free capsomers; a/s = amorphous substance; SRP = small roundish particles; - = not present or not described; * = particularly pronounced

Table A.3a. Sizes of papovaviruses - papillomaviruses

Virus	Prep	Cal	H/G	Size	No	Range	Comments	Reference
Shope	AD	TMV	-	44 nm	21	-	spherical isolated, mixed with TMV	Sharp et al, 1942
	SP	-	-	66 nm	-	-	spherical isolated, flattened	Sharp et al, 1946
	TS	-	-	-	-	26-29 nm	in spherical	Haguenau et al, 1960
	NS	-	-	-	-	55-58 nm	spherical	Breedis et al, 1962
	NS	-	-	53.7 nm	50	-	spherical full, mixed with polyoma	Crawford & Crawford, 1963
	NS	-	-	-	-	45-55 nm	spherical + 30 nm filaments	Howatson & Crawford, 1963
	NS	-	-	-	-	53-57 nm	spherical full + 30 & 45-55 nm filaments	Finch & Klug, 1965
	NS	-	-	50 nm	-	-	+ 38 nm spherical empty	Finch & Klug, 1965
human	SP	-	-	52 nm	-	50-53 nm	spherical packed	Bunting, 1953
	SP	-	-	68 nm	-	56-80 nm	spherical isolated	Bunting, 1953
	SP	-	-	55 nm	-	-	spherical close packed, 65-75 nm isolated	Williams et al, 1961
	SP	-	-	60 nm	-	-	spherical isolated	Smith et al, 1965
	PS	-	-	49 nm	-	-	spherical isolated	Smith et al, 1965
	TS	-	-	46 nm	-	-	in spherical	Williams et al, 1961
	NS	-	-	52.4 nm	50	-	spherical full, mixed with polyoma	Crawford & Crawford, 1963
	NS	-	-	53 nm	-	-	spherical empty	Noyes, 1964
	NS	-	-	57 nm	-	-	spherical full + 40 nm filaments	Noyes, 1964
	NS	-	-	49 nm	-	-	spherical isolated, empty	Klug & Finch, 1965
	NS	-	-	53 nm	-	-	spherical isolated, full	Klug & Finch, 1965
	NS	-	-	55 nm	-	-	spherical	Gardner, 1977
	NS	-	his	58.8 nm	33	55-63 nm	spherical, human papilloma in urine	Lecatsas, Boes & Horsthemke, 1981
	NS	cat	-	57.1 nm	100	53-60 nm	spherical	Richmond et al, 1984
bovine	NS	-	-	52 nm	30	-	spherical full, mixed with polyoma	Crawford & Crawford, 1963
canine	NS	-	-	54.7 nm	50	-	spherical full, mixed with polyoma	Crawford & Crawford, 1963

continued ...

For table abbreviations, please see A.3g

Table A.3b. Sizes of papovaviruses - polyomaviruses

Virus	Prep	Cal	H/G	Size	No	Range	Comments	Reference
K virus	TS	-	-	30 nm	-	-	in & ic spherical	Dalton et al, 1959
	TS	rep	-	35 nm	-	-	+ few up to 45 nm in spherical	Dalton et al, 1963
	TS	-	-	42 nm	-	-	in spherical	Greenlee et al, 1982
	NS	-	-	50 nm	-	-	spherical	Mattern et al, 1963
	NS	rep	his	42 nm	100	38-52 nm	spherical full	Parsons, 1963
polyoma	TS	-	-	-	-	27-35 nm	in & ic spherical	Banfield et al, 1959
	TS	-	-	30 nm	-	26.5-32.2 nm	in spherical, ic 40-60 nm	Bernhard et al, 1959
	TS	-	-	-	-	30-40 nm	in spherical, ic 50-60 nm with membrane	Negroni et al, 1959
	TS	-	-	28 nm	-	-	in spherical + 32 nm filaments	Howatson & Almeida, 1960a
	TS	-	-	38 nm	-	-	in spherical + 32 nm filaments	Howatson & Almeida, 1960b
	TS	-	-	60 nm	-	-	ic with membrane	Howatson & Almeida, 1960b
	TS	-	-	28 nm	-	-	in spherical, ic 60 nm with membrane	Howatson et al, 1960
	TS	rep	-	35 nm	-	-	+ some up to 45 nm in spherical	Dalton et al, 1963
	TS	-	-	-	-	15-38 nm	in filaments	Mattern et al, 1966
	TS	-	-	38 nm	-	-	in spherical, ic 50-60 nm with membrane	Mattern et al, 1966
	TS	-	-	-	-	35-40 nm	in spherical	Sebesteny et al, 1980
	TS	-	-	38 nm	-	-	in spherical	Taddei et al, 1982
	TS	-	-	-	-	35-40 nm	in spherical	McCance et al, 1983
	NS	-	-	45 nm	-	-	spherical + 38 nm filaments	Howatson & Almeida, 1960b
	NS	-	his	42.8 nm	49	41-45 nm	spherical empty	Wildy et al, 1960
	NS	-	his	44.7 nm	94	42-47 nm	spherical full	Wildy et al, 1960
	NS	-	his	50 nm	90	47-52 nm	spherical, partially disrupted	Wildy et al, 1960
	NS	-	-	43 nm	50	-	spherical full, mixed with human papilloma	Crawford & Crawford, 1963
	NS	-	-	43.5 nm	50	-	spherical full, mixed with bovine papilloma	Crawford & Crawford, 1963

continued ...

Table A.3c. Sizes of papovaviruses - polyomaviruses

Virus	Prep	Cal	H/G	Size	No	Range	Comments	Reference
polyoma	NS	-	-	43.7 nm	50	-	spherical full, mixed with Shope papilloma	Crawford & Crawford, 1963
(contd)	NS	-	-	44.3 nm	50	-	spherical full, mixed with canine papilloma	Crawford & Crawford, 1963
	NS	rep	his	38.8 nm	35	36-42 nm	spherical empty	Parsons, 1963
	NS	rep	his	47 nm	30	42-54 nm	spherical full	Parsons, 1963
	NS	-	-	48 nm	-	-	+ 22 & 38 nm spherical	Mattern et al, 1967
	NS	-	his	45 nm	200	30-75 nm	spherical	Lecatsas & Mallucci, 1972
	NS	-	his	45 nm	36	15-70 nm	filaments	Lecatsas & Mallucci, 1972
	NS	MV	-	43 nm	-	41.5-44.5 nm	spherical	Seehafer et al, 1975
SV40	SP	rep	-	44.1 nm	-	43.5-44.7 nm	in spherical	Koch et al, 1967
	TS	-	-	30 nm	-	27-33 nm	in spherical	Gaylord & Hsiung, 1961
	TS	-	-	40 nm	-	-	in spherical	Mayor et al, 1962
	TS	-	-	33 nm	-	-	in spherical, ic 50 nm with membrane	Granboulan et al, 1963
	TS	-	-	45 nm	-	-	in spherical	Petursson et al, 1966
	TS	-	-	45 nm	-	-	in spherical	Sheffield et al, 1980
	NS	-	-	45 nm	100	43.5-46.5 nm	spherical	Bernhard et al, 1962
	NS	-	-	45 nm	-	-	spherical	Mayor et al, 1963
	NS	-	-	-	-	42-45 nm	spherical, mixed with human papilloma	Black et al, 1964
	NS	rep	-	16.8 nm	-	15.8-17.8 nm	spherical	Anderer et al, 1967
	NS	rep	-	27.4 nm	-	26.5-28.3 nm	spherical	Anderer et al, 1967
	NS	rep	-	31.5 nm	-	29.8-33.2 nm	spherical	Anderer et al, 1967
	NS	rep	-	42 nm	-	40.3-43.7 nm	spherical	Anderer et al, 1967
	NS	rep	-	42 nm	200	40.3-43.7 nm	spherical empty	Koch et al, 1967
	NS	rep	-	45 nm	23	43.3-46.7 nm	spherical isolated	Koch et al, 1967

continued ...

Table A.3d. Sizes of papovaviruses - polyomaviruses

Virus	Prep	Cal	H/G	Size	No	Range	Comments	Reference
SA12	TS	-	-	45 nm	-	-	in spherical, ic 53 nm with membrane	Malherbe & Strickland-Cholmley,
	NS	-	-	43 nm	-	-	spherical	Lecatsas et al, 1977 (1973)
	NS	-	-	-	-	44-45 nm	spherical	Valis et al, 1977
RKV	TS	-	-	28 nm	-	35-42 nm	in filaments	Chambers et al, 1966
	TS	-	-	38 nm	-	35-42 nm	in spherical, ic 47 nm with membrane	Chambers et al, 1966
	NS	-	-	47 nm	431	42.5-51.5 nm	spherical + 37-40 nm filaments	Chambers et al, 1966
PML	TS	-	-	38 nm	-	-	in spherical + narrower filaments	Howatson et al, 1965
	TS	-	-	-	-	35-40 nm	in spherical + 35-40 nm filaments	Silverman & Rubinstein, 1965
	TS	-	-	-	-	33-36 nm	in spherical + 16-24 nm filaments	ZuRhein & Chou, 1965
	TS	-	-	-	-	15-25 nm	in filaments	ZuRhein, 1969
	TS	-	-	-	-	28-40 nm	in spherical most 33-38 nm	ZuRhein, 1969
	NS	-	-	40.5 nm	-	-	spherical, fixed brain	Howatson et al, 1965
	NS	-	-	41 nm	50	-	spherical	Howatson et al, 1965
	NS	-	-	42.5 nm	-	-	spherical, unfixed brain	Howatson et al, 1965
	NS	-	-	38 nm	20	-	spherical, larger virions damaged	Schwerdt et al, 1966
JCV	TS	-	-	42.5 nm	-	-	in spherical + 29 nm filaments	Padgett et al, 1971
	NS	-	-	42.3 nm	50	-	spherical, culture	Padgett et al, 1971
	NS	-	-	43.5 nm	-	-	spherical, brain	Padgett et al, 1971
	NS	-	-	-	-	38-42 nm	spherical + 40 nm filaments	Albert & ZuRhein, 1974
SV40-PML	TS	-	-	-	-	33-35 nm	in spherical + 15 nm filaments	Weiner et al, 1972
	NS	-	-	42 nm	-	-	spherical	Weiner et al, 1972
	NS	-	-	-	-	38-42 nm	spherical, SV40-PML	Albert & ZuRhein, 1974

continued ...

Table A.3e. Sizes of papovaviruses - polyomaviruses

Virus	Prep	Cal	H/G	Size	No	Range	Comments	Reference
JCV-COL	TS	-	-	39.1 nm	-	-	in spherical + 23 nm filaments, brain	Field et al, 1974
	TS	-	-	43.4 nm	-	-	in spherical + 23 nm filaments, culture	Field et al, 1974
	NS	-	-	39.1 nm	-	-	spherical, brain	Field et al, 1974
	NS	-	-	49.3 nm	-	38.2-56.9 nm	spherical, larger virions damaged, culture	Field et al, 1974
FPV	TS	-	-	-	-	40-50 nm	in spherical	Lunger, 1966
	NS	-	-	45 nm	-	-	spherical	Granoff, 1969
HPV/LHV	TS	-	-	35 nm	-	-	in spherical	Graffi et al, 1968
	TS	-	-	40 nm	-	35-40 nm	in spherical	Hannoun et al, 1974
BKV	NS	-	-	43.6 nm	-	-	spherical	Gardner et al, 1971
	NS	-	-	-	-	41-44 nm	spherical + 40 nm filaments	Albert & ZuRhein, 1974
	NS	-	-	43 nm	-	-	spherical + 30 & 43 nm filaments	Lecatsas & Prozesky, 1975
	NS	MV	-	40.5 nm	-	39.5-41.5 nm	spherical	Seehafer et al, 1975
	NS	-	-	43.6 nm	175	-	spherical	Gardner, 1977
	NS	-	his	39 nm	50	37-43 nm	spherical	Lecatsas et al, 1981
	NS	-	his	41 & 52	100	33-59 nm	spherical, polyoma/papilloma intermediate	Lecatsas et al, 1981

continued ...

Table A.3f. Sizes of papovaviruses - polyomaviruses

Virus	Prep	Cal	H/G	Size	No	Range	Comments	Reference
SPV	NS	-	-	35.8 nm	-	-	filaments	Newman & Smith, 1972
	NS	-	gra	39.6 nm	103	32-56 nm	spherical, 80% of virions 36-44 nm	Newman & Smith, 1972
	NS	-	-	40.5 nm	-	39-42 nm	spherical	Tischer et al, 1974
	NS	-	-	41.2 nm	101	-	spherical, SPV	Tumilowicz et al, 1979
	NS	-	-	42.6 nm	104	-	spherical, CCL 33 PV	Tumilowicz et al, 1979
OMKV	TS	-	-	-	-	39-40 nm	in spherical	King et al, 1972
STMV	TS	-	-	35 nm	-	-	in spherical, ic 55 nm with membrane	Rangan et al, 1974
	NS	-	-	-	-	40-45 nm	spherical 55-60 nm with membrane	Reissig et al, 1976
M-PML	TS	-	-	30 nm	-	-	in spherical	Gribble et al, 1975
WRSV	TS	-	-	35 nm	-	-	in spherical, ic 45-50 nm with membrane	Coackley et al, 1980
	NS	-	-	45 nm	-	-	spherical	Coackley et al, 1980

continued ...

Table A.3g. Sizes of papovaviruses - polyomaviruses

Virus	Prep	Cal	H/G	Size	No	Range	Comments	Reference
BFDV	TS	-	-	-	-	42-49 nm	in spherical	Bozeman et al, 1981
	TS	-	-	-	-	42-49 nm	in spherical	Davis et al, 1981
	TS	-	-	42 nm	-	-	in spherical	Jacobson et al, 1984
	NS	-	-	-	-	50-55 nm	spherical	Bernier et al, 1981
	NS	-	-	-	-	42-49 nm	spherical	Dykstra & Bozeman, 1982
	NS	-	-	-	-	46-48 nm	spherical	Muller & Nitschke, 1986
FRKV	NS	cat	-	46.8 nm	100	44-50 nm	spherical	Richmond et al, 1984
RPV	TS	-	-	45 nm	-	-	in spherical	Ward et al, 1984
CKV	NS	-	-	-	-	35-45 nm	spherical 48 nm with membrane	Westcott et al, 1987
PP-2	NS	-	-	47.9 nm	93	41.5-56.5 nm	spherical, 50% of virions 45-49 nm	Gardner et al, 1989

Prep = preparation: AD = air dried; SP = shadow preparation; TS = thin section; NS = negative stain; PS = positive stain

Cal = calibration of microscope magnification: - = not stated; cat = crystalline catalase; rep = replica; MV = Mengo virus

H/G = histogram or graph of measurements; - = neither; his = histogram; gra = graph. No = number of particles measured: - = not stated

Comments: TMV = tobacco mosaic virus; in = intranuclear; ic = intracytoplasmic; full = not penetrated by negative stain; empty = penetrated by negative stain

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Immunoelectron Microscopy of the Papovavirus from Two Foetal Rhesus Monkey Kidney Cell Lines used for Hepatitis A Virus Propagation.

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There have been several reports of the growth of hepatitis A virus in the foetal rhesus monkey kidney cell lines FRhK-4 and FRhK-6. In addition, FRhK-6 cells are being used in the preparation of a live attenuated hepatitis A vaccine.

We have detected a papovavirus in both of these cell lines by electron microscopy. The virus belongs to the polyomavirus genus and has been successfully grown in primary human embryo kidney cells.

Preliminary immunoelectron microscopy (IEM) indicates that the virus is not the simian polyomaviruses SV40 or SA12, nor the human polyomaviruses BK or JC. The virus reacts with an antibody-like structure present in some bovine sera and in antisera raised against STMV, the papovavirus isolated from stump-tailed macaque kidney cell cultures.

The results of these and further IEM studies will be presented.

POLYOMAVIRUS IN FETAL RHESUS MONKEY KIDNEY CELL LINES USED TO GROW HEPATITIS A VIRUS

SIR,—Fetal rhesus monkey cell lines have been used to propagate hepatitis A virus (HAV) both for laboratory use[1] and vaccine development.[2] We obtained one such cell line, FRhK-4 (pass 76), from Dr B. Flehmig, Tübingen University, West Germany, to investigate its suitability for producing HAV antigen for use as a diagnostic reagent. When tissue culture fluids from HAV inoculated, uninoculated control, and stock cultures, incubated for prolonged periods, were examined by electron microscopy (EM) virions with the morphology and size characteristic of polyomaviruses were seen (figure).

To determine whether or not this virus was a recent contaminant, lower passage FRhK-4 cultures (pass 25), together with the FRhK-6 cell line (pass 6), were obtained from Dr J. C. Petricciani, Bureau of Biologics, National Institutes of Health, Bethesda, Maryland, USA, where these cell lines had originally been developed and characterised.[3] After prolonged incubation, during which precautions were taken to avoid cross-contamination, polyomavirus particles were detected in both of these cell lines by EM. Examination of the FRhK-4 cells by an indirect immunofluorescence (IF) technique showed that less than 10% of cells were positive at any time. Light microscopy demonstrated only minor cytopathic changes.

The FRhK-4-derived polyomavirus induced cytopathic changes in human embryonic kidney cultures (HEK), and viral replication was confirmed by IF and EM. Immunoelectronmicroscopy (IEM) has shown that the FRhK-4 virus is not one of the primate polyomaviruses SV40, SA12, BK or JC. The virus does, however, react with antisera raised against the stump-tailed macaque virus (STMV) by IEM, IF, and countercurrent immunoelectrophoresis (CIE). (The STMV and SA12 antisera were kindly provided by Dr K. Shah, Johns Hopkins University, Baltimore, Maryland, USA.) Examination of sera from 45 rhesus monkeys and 97 human blood donors by CIE has not revealed the presence of antibody to this virus.

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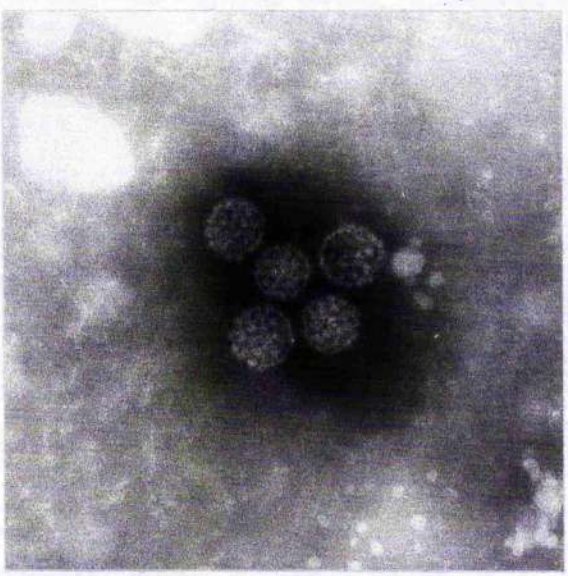

Polyomavirus particles from culture fluid of FRhK-4 cells incubated for 42 days, stained with 3% phosphotungstic acid (× 185 000).

However, some fetal and newborn bovine sera contain an antibody-like substance which coats the virus (EM and IEM), and the presence of antibody in 48% of 353 cattle sera has been demonstrated by CIE. This suggests that the FRhK-4-derived polyomavirus may be bovine in origin. More detailed reports are in preparation.

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INCREASED URINARY EXCRETION OF A GLYCOGEN-DERIVED TETRASACCHARIDE IN HETEROZYGOTES WITH GLYCOGEN STORAGE DISEASES TYPE II AND III

SIR,—A glucose tetrasaccharide (Glc 1-6Glc 1-4Glc 1-4Glc), denoted $(Glc)_4$, appears normally in human urine in amounts within the range 0·1–2·5 mg in 24 h.[1] The rate of excretion of $(Glc)_4$ is greater in various clinical conditions associated with increased storage or turnover of glycogen, of which glycogenosis,[1,2] Duchenne muscular dystrophy,[3] and pregnancy[4,5] are examples.

$(Glc)_4$ can be formed by amylolytic degradation of glycogen.[6,7] The amount of $(Glc)_4$ normally excreted in urine per 24 h could arise from intravascular degradation of about 30 mg glycogen, which represents 0·01% of the total body glycogen content.[7] Intravenous injection of 100 mg glycogen into a rhesus monkey resulted in a urinary excretion of 7 mg $(Glc)_4$ within 5 h with maximum excretion being attained 10 min after the injection (unpublished). All these data suggest that the urinary excretion rate of $(Glc)_4$ is a sensitive indicator of increased release of glycogen from cells into the circulation.

The highest excretion rates of $(Glc)_4$ have been observed in patients with glycogen storage disease (GSD) II and III. These patients have a large intracellular accumulation of glycogen and, presumably, increased release of glycogen into the circulation. Histochemical and ultrastructural studies on muscle biopsy material from GSD II heterozygotes did not indicate any increased accumulation of glycogen,[8] but in GSD III heterozygotes the glycogen content of erythrocytes is increased to a range intermediate between normals and affected individuals.[9] Since enzymatic identification of GSD heterozygotes has several limitations[8] we have evaluated urinary $(Glc)_4$ determination as a possible diagnostic aid.

24 h urine specimens were collected from eight GSD II patients and one GSD III patient. Urine was also collected from seventeen healthy members of families having at least one member with either GSD II or III. $(Glc)_4$ was determined by radioimmunoassay,[5] and the results (see table) were confirmed by gas chromatography/mass spectrometry.[1]

24 h urinary excretion of $(Glc)_4$ was increased to 9·9–166 mg in the patients with GSD II or III. These data confirm previous observations.[2] All but two of the ten obligate heterozygotes (parents)

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Evidence for a Bovine Origin of the Polyomavirus Detected in Foetal Rhesus Monkey Kidney Cells, FRhK-4 and -6

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With 2 Figures

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Summary

Rabbit antisera to the stump-tailed macaque polyomavirus (STMV) which had been shown by immunoelectron microscopy and indirect immunofluorescence to react with the polyomavirus found in FRhK-4 cells (FRKV), also gave precipitin lines in counter-immunoelectrophoresis (CIE) and double diffusion in gel (GD) when reacted with FRKV. The reactions in GD showed identity with that of a rabbit antiserum to FRKV.

Naturally occurring antibody to FRKV (anti-FRKV) was found by CIE in 48 per cent of 353 cattle, 1/106 pigs and 1/20 goats but not in any of 13 other species including 45 rhesus monkeys and 97 humans. Each of 9 anti-FRKV positive samples from cattle, the goat serum, but not the pig serum gave a line of identity with the rabbit antiserum to FRKV in GD against FRKV. Detection of anti-FRKV in colostrum deprived newborn calves and in commercial foetal calf sera (FBS) indicates that intra-uterine infection of cattle with FRKV may occur.

FRKV adapted readily to growth in secondary calf kidney cultures and grew more rapidly and to higher titres than in the FRhK-4 cultures.

We conclude that FRKV is probably another strain of STMV and that the natural hosts of these viruses are cattle and not primates. Evidence of intra-uterine infection of cattle implies that infectious FRKV may be present in some FBS and may thus have gained entry into various susceptible cell lines, particularly primate kidney.

Introduction

The discovery of an adventitious polyomavirus in the FRhK-4 cell line and subsequently in a similar cell line. FRhK-6 has been reported by us (6, 7). These

two cell lines are representatives of several developed by Wallace and her colleagues (15). One of these cell lines, FRhK-6, is known to have been used in the development of a live attenuated hepatitis A vaccine (8).

Many of the known polyomaviruses of mammals were primarily, and in some cases, solely isolated from cell cultures derived from kidney tissue (Table 1). Evidence of natural infection with the isolated polyomaviruses has been demonstrated in the presumed host species by the presence of serum antibody to that virus, except for the stump-tailed macaque virus (STMV) (9) and HD virus (14), the latter present in a cell line, Vero, derived from African green monkey kidney. These two viruses have subsequently been shown to be indistinguishable (4).

Table 1. *Host, origin and evidence of host antibody for the known polyomaviruses*

Virus	Reported host	Original isolation from	Naturally occurring host antibody
polyoma	Mouse	Pooled tissues	Positive
K	Mouse	Pooled tissues	Positive
LHV	Hamster	Spleen/kidney	Not known
RKV	Rabbit	Cutaneous papilloma[a]	Positive
SPV	Pig	Kidney cultures	Positive
WRSV	Cattle	Kidney cultures	Not known
LPV	African green monkey	Lymphoblastoid culture	Positive
SA12	African green monkey[b]	Kidney cultures	Positive
SV40	Rhesus monkey	Kidney cultures	Positive
STMV	Stump-tailed macaque	Kidney cultures	Negative
BK	Man	Urine	Positive
JC	Man	Brain	Positive

[a] Isolated simultaneously with a papillomavirus in rabbit kidney cultures
[b] Chacma baboon is the principal host

Immunoelectron microscopy (IEM) and indirect immunofluorescence (IF) have shown that the FRhK-derived virus (FRKV) is identical or closely related to STMV and is not one of the polyomaviruses SV40, SA12, BK or JC (10). As reported here the presence of antibody to FRKV (anti-FRKV) was not demonstrable in rhesus monkeys. It therefore seems likely that FRKV may be a further strain of the STMV/HD type. On the basis of the STMV model the natural conclusion would be that FRKV is also of primate origin. However, the observation was made by electron microscopy (EM) that free polyomavirions prepared from clarified tissue culture fluid (TCF) removed from FRhK-4 cultures were coated with a material morphologically indistinguishable from antibody, whereas virions from disrupted washed FRhK-4 cells were not antibody coated (10). The observation of unidentified structures attached to polyomavirions prepared from TCF is not novel (9, 13) and has been associated specifically with the bovine serum in the TCF (13). However, the possibility that, in addition to these observations, a virus-antibody reaction might also be present appears not to have been investigated. Our findings (10) have led us to investigate the possibility that one of the reacting components of bovine sera is antibody and that FRKV may be of bovine origin (7).

A technique for detecting antibody which was less laborious than IEM, but still applicable to serum from any species was sought. As it was not possible to demonstrate haemagglutination by this polyomavirus (10), counter-immuno-electrophoresis (CIE) and double diffusion in gel (GD) were investigated and found to be satisfactory. The results of a search for the presence of naturally acquired anti-FRKV by CIE in a variety of species are presented in this paper.

The discovery and characterisation of FRKV may not only be of consequence for the experimental attenuated hepatitis A vaccine for which the virus had undergone several passages in FRhK-6 cultures (8); the results presented below lead to conclusions which have far wider implications in the field of vaccine development. In addition, the description of STMV as a virus of stump-tailed macaques and its congenital transmission (11) must now be reconsidered.

Materials and Methods

Cell Culture

Cell Cultivation

i) FRhK-4: These cultures had been obtained from Dr. B. Flehmig, Tübingen University, Germany at passage 76 and were used up to passage 94 for these investigations. The cells were grown at 37° C on Eagles minimum essential medium (MEM) containing 6 per cent foetal bovine serum (FBS). Confluent cultures were incubated at 33° C on MEM with 2 per cent FBS which was replenished at weekly intervals. Cell stripping was carried out using phosphate buffered saline A (PBS) containing trypsin and versene.

ii) Calf Kidney (CK): Primary cultures were treated in a similar way to the FRhK-4 cultures except that 10 per cent FBS was used in the growth medium. Secondary cultures were prepared in 250 ml flasks. One flask from each batch of secondary cultures was set aside for long-term incubation in an attempt to isolate a polyomavirus directly from calf kidney cell culture. The cultures were maintained for part of the time on serum-free medium, Iscoves modification of Dulbecco's MEM, complete medium (Flow Labs.) and part of the time on MEM with 2 per cent FBS. Preparations of low-speed pellets from the TCF collected from these cultures were negatively stained with 3 per cent phosphotungstic acid, pH 6.3, and examined by EM.

Virus Propagation

i) FRhK-4: Because many commercially available pooled bovine sera preparations appeared to possess antibody (10 and below), the chronically infected cultures were incubated with Iscoves medium. The confluent cultures were washed twice with PBS before receiving the Iscoves medium. The cultures were then incubated stationary at 37° C with medium changes at two week intervals.

ii) CK Cultures: Passage 1: Two flasks of confluent 2° CK were washed with PBS. One flask, the control, received 20 ml of Iscoves medium alone; the other received 20 ml of clarified Iscoves medium harvested from 2 cultures of FRhK-4 cells which had been seeded 12 weeks previously and had received their last medium change 2 weeks earlier. The CK cultures were incubated at 37° C and the medium was replenished at days 5 and 13. The TCF and cell sheets were harvested together at day 27.

Passage 2: Three flasks of confluent 2° CK were washed with PBS and then inoculated with 1.5 ml of frozen and thawed harvest from passage 1 at day 27. After adsorption for 3 hours at 37° C, 20 ml of fresh Iscoves medium was added to each and incubation continued for 17 days at 33° C. The medium was replenished at day 14. Uninoculated control flasks of the same 2° CK batch were incubated simultaneously.

Passage 3: Two flasks received 7.5 ml each of clarified TCF, pooled from days 14 and 17 of passage 2. After adsorption the inoculum was supplemented by 15 ml of Serumless Medium (Gibco) with 2 per cent gammaglobulin-free equine serum (EqS). The TCF was replenished at days 8 and 13 and the passage terminated at day 15.

Polyomavirus Antigen Preparation

a) Crude Preparation: It had been noted that the cells which are released into the TCF yielded most virus (10). Therefore, cells were pelleted from TCF by centrifugation at $1500 \times g$ for 10 minutes, resuspended in a small volume of water and sonicated in a bath sonicator to release the virus. The sonicates were used as FRKV antigen in some of the preliminary CIE tests.

b) Semi-purified Preparation: Sonicates of cell pellets were prepared as in a). Larger particulate material was removed by low-speed centrifugation and then 0.5 ml of each supernatant placed on a density gradient composed of 30, 40, 50 and 60 per cent weight/volume sucrose in distilled water which had been allowed to equilibrate. The gradients were centrifuged at $250,000 \times g$ for 4 hours in a swing-out rotor and then 14—18 fractions, each of 5 drops collected from each of them. The fractions were tested for FRKV by CIE (see below) and those giving a specific reaction pooled for use as antigen.

Counter-Immunoelectrophoresis (CIE)

The method used was that described by Cohen *et al.* (2). The polyomavirus antigen was used at 4—8 times its CIE end point. Newborn calf serum, NBS 22/6, which was positive for anti-FRKV by IEM, CIE and GD was designated the antibody positive control for CIE and was included at least once on every CIE plate used for antibody testing. NBS 22/6 was also used as the antiserum in CIE tests for the detection of FRKV antigen. Gels were examined for precipitin lines which were scored on a scale 0 to ++++.

EM Examination of Precipitin Lines

A sample of precipitin lines was selected from the various groups of sera tested by CIE and examined by EM for virus-antibody reactions. The lines were carefully cut out, homogenised in 20—40 µl of water, applied to formvar/carbon-coated grids and negatively stained as above.

Double-Diffusion in Gel

Wells (3 mm) were cut in a hexagonal pattern around a central well. The gel consisted of 0.75 per cent agarose in a barbitone buffer of which 3 ml had been poured into a 35 mm plastic petri dish. The virus preparation was placed in the central well and the sera in the outer wells. The wells were refilled after 4 hours and the gels examined for precipitin lines 24—48 hours later.

Receptor Destroying Enzyme (RDE) Treatment

Serum samples from cattle were mixed with RDE (Wellcome Reagents Ltd.) in the proportion of 1 part to 4 parts, incubated at 37° C overnight and then inactivated at 56° C for 1 hour. The experiment was controlled by treating duplicate samples similarly except that saline was substituted for RDE. Both samples were then tested for anti-FRKV by CIE.

Immunoglobulin G (IgG) Purification

Immunoglobulin was precipitated by ammonium sulphate from two cattle sera that contained anti-FRKV. The immunoglobulin from one sample, NBS 22/6, was then further purified both by affinity chromatography, utilising protein A-sepharose and by an ion exchange technique using DE 52 (Whatman) under IgG eluting conditions of 50 mM phosphate buffer, pH 6.3. The second serum, BW 14467, was not processed by the protein A technique. The protein concentrations of the purified IgG were determined by optical density measurement of the samples at 280 nm and then adjusted to approximately 10 mg/ml.

Results

Virus Propagation and Antigen Production

FRhK-4 Cultures

Sufficient quantities of FRKV antigen for use in CIE were not detected until at least 6 weeks after seeding the FRhK-4 cultures. Table 2a shows the antigen titres in CIE of a series of different cell culture preparations. Although 4 preparations, 1. 3. 5 and 8 were sufficiently potent for CIE. two of these, 5 and 8, were of poor quality. and gave rise to indistinct non-specific precipitation which masked the sharp specific lines of the virus-antibody reaction. These non-specific reactions increased as the antigen aged and were soluble upon soaking the CIE gel in PBS. whereas the specific precipitin lines were unaffected. The FRKV antigen preparations which were unsuitable for direct use in CIE were combined (FRhK-4 Pool) before concentration and purification.

Table 2. *Reactions of crude FRKV antigen preparations with anti-FRKV in CIE*

a) *FRhK-4 cultures*

FRKV antigen preparation	Results of antigen titration in CIE versus NBS 22/6 (neat)						Application
	Undil.	1/2	1/4	1/8	1/16	1/32	
1	+++	+++	+	±	—	—	CIE
2	+++	++	±	—	—	—	FRhK-4 pool
3	+++	+++	++	+	±	—	CIE
4	++	+	—	—	—	—	FRhK-4 pool
5	+++	++	—	±	—	—	FRhK-4 pool
6	+++	±	—	—	—	—	FRhK-4 pool
7	+++	±	—	—	—	—	FRhK-4 pool
8	+++	+	—	±	—	—	FRhK-4 pool

b) *Secondary calf kidney*							Comments
CK 1/CON	—	—	—	—	—	—	uninoculated control day 27
CK 1/FRKV	+++	+++	—	±	±	—	FRKV passage 1 in CK, day 27
CK 2/FRKV	+++	+++	+++	+++	++	+	FRKV passage 2 in CK, day 17

Purification of the FRKV antigen on SDG separated the material which causes the non-specific precipitation; this remained near the top of the gradient, whereas the viral antigen migrated through about two-thirds of the gradient (Table 3a). The pooled CIE positive fractions had undergone approximately 16-fold concentration by volume from the starting material, the FRhK-4 Pool, to yield a sufficiently potent CIE antigen (Table 3b). Non-specific activity could not be detected by CIE in the SDG purified antigen.

Table 3. *Semipurification of FRKV from cell homogenates of FRhK-4 and CK cultures*

a) *CIE test results on sucrose density gradient (SDG) fractions*

	Gradient fraction													
	Heavy →													→ Light
Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14
FRhK-4 pool	—	—	+	+	+	++	+	—	—	—	—	±	+	++
CK2/FRKV	—	—	+	++	++	++	+	—	—	—	±	±	±	+
CK1/control	—	—	—	—	—	—	—	—	±	±	±	±	—	—

b) *CIE titre of SDG — purified FRKV pools*

	Results of antigen Titration in CIE versus NBS 22/6 (neat)					
Sample	Undil.	1/2	1/4	1/8	1/16	1/32
CK1/control (1×)	—	—	—	—	—	—
CK1/CK2 pool FRKV (1×)	++	+	+	±	—	—
FRhK-4 pool (16×)	++	+	+	±	—	—

Calf Kidney Cultures

FRKV was found to adapt readily to calf kidney cultures. Widespread involvement of the cell sheet occurred in successively shorter periods at each of the first 3 passages: 27 days, 17 days and 15 days. In addition, areas of the typical polyomavirus cytopathic effect (CPE) of cytoplasmic vacuolation became more extensive at each passage. The uninoculated control cultures remained unchanged. Fig. 1 shows the appearance of the CPE in a CK culture on day 17 of the third passage of FRKV in CK cultures and that of the control culture. The yield of viral antigen by CIE is given in Table 2b for passages 1 and 2 and a control culture. These results confirmed the impression given by microscopic examination of cultures for CPE.

The crude FRKV antigen from CK cultures was of higher titre and did not produce the non-specific reactions shown by the FRhK-4 product. Purification by SDG was however carried out. Banding of the viral antigen in the gradient was similar to the FRhK-4 derived antigen (Table 3a) and the pooled SDG fractions gave an identical potency without the necessity of concentration (Table 3b). The control preparation showed traces of non-specific precipitation near the top of the gradient.

Examination by EM of disrupted cell pellets from the TCF of 7 different batches of 2° CK which are on long-term incubation has failed to reveal the presence of polyomavirions. The cultures had been maintained for between 16 and 69 days since seeding.

Identity of FRKV

FRKV gave specific precipitin lines when tested in CIE against two rabbit antisera to the polyomavirus STMV, kindly donated by Dr. K. V. Shah, Johns

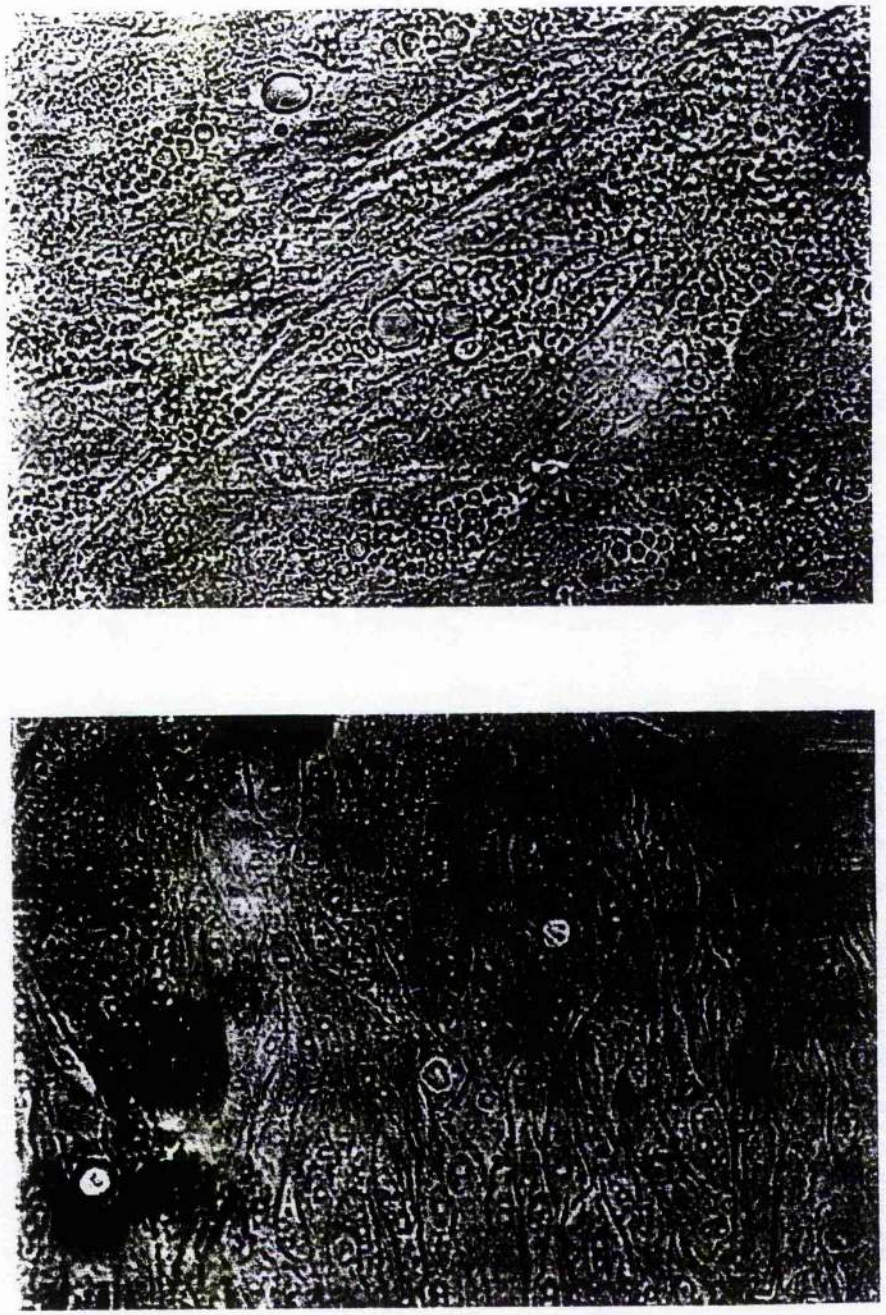

Fig. 1. Characteristic polyomavirus cytopathic effect caused by FRKV infection of secondary calf kidney cells after 15 days incubation (top) and the uninfected control cells (bottom). $\times 330$

Hopkins University, Baltimore, U.S.A., and against a rabbit antiserum to FRKV prepared in this laboratory. Antisera against polyomaviruses BK and JC, which gave precipitin lines against their homologous antigen, did not precipitate FRKV in CIE.

When STMV and FRKV antisera were tested in adjacent wells of a double diffusion in gel test against the purified FRKV antigen, a line of identity resulted. Such a line of identity was observed with each of the STMV rabbit antisera, 75—176 and 81—239. It was not possible to perform this test against the STMV antigen as this was not available.

Verification of a Virus-Antibody Reaction

The three investigations carried out to confirm that the virus precipitation detected by CIE was mediated by antibody gave results which were compatible with this type of reaction. Firstly, immunoglobulin G which was purified from cattle sera by the standard techniques outlined above continued to precipitate FRKV. Secondly, identical reactions were obtained from serum samples which were RDE treated to remove any non-specific inhibitors and from the same samples which received the control treatment. Finally, EM examination for polyomavirions coated with antibody in the CIE precipitin lines formed between the FRKV antigen and the following bovine samples confirmed a virus-antibody reaction: 2 NBS, 5 individual bovine sera, 2 IgG preparations purified from NBS, 2 RDE treated NBS and 1 FBS. Two additional FBS samples gave rise to equivocal EM results since only small amounts of antibody were observed attached to the virions.

Serological Surveys by CIE

Screening of Various Animal Species for Anti-FRKV

Only a few of the species listed in Table 4 were found to possess antibody to FRKV. Forty-eight per cent of sera from cattle collected from a number of herds were found to be positive. The one goat found to be seropositive was bled at the same time as 3 others from the same herd which were negative. The pig which was positive was the only one out of 7 adults from the same herd. Anti-FRKV was not found in any primates examined, including 45 rhesus monkeys and 97 human blood donors.

Detailed Analysis of CIE Results on Cattle Sera

The distribution of anti-FRKV in cattle relative to their age is shown in Fig. 2. Forty per cent of newborn calves were seropositive. At 12 months only 11 per cent were definitely positive and thereafter an upward trend towards a very high proportion of seropositives was noted. Of the 273 cattle sera represented in Fig. 2, 230 were known to be from the females of 11 herds of Friesians or Holsteins in England and Wales. The proportion of anti-FRKV positive cattle for each herd varied between 24 and 75 per cent with a mean of 52 per cent. The prevalence of seropositivity seemed to bear no relationship to the area of the country in which the herd was kept.

Table 4. *Results of screening for the presence of anti-FRKV by CIE in 766 sera collected from 16 animal species*

Source of sera	No. tested	No. positive	No. equivocal
Fowl	5	—	—
Pigeon	5	—	—
Mouse	22	—	—
Rat	10	—	—
Rabbit	14	—	—
Hamster	7	—	—
Guinea pig	12	—	—
Pig	106	1	—
Sheep[a]	27	—	1
Goat	20	1	—
Cattle	353	170	12
Marmoset	12	—	—
Baboon	25	—	—
Rhesus monkey[b]	45	—	—
Assorted primates	6	—	—
Human (blood donor)	97	—	—

[a] 7 of these animals had been used to prepare hyperimmune sera to echoviruses
[b] One animal immunised against malaria

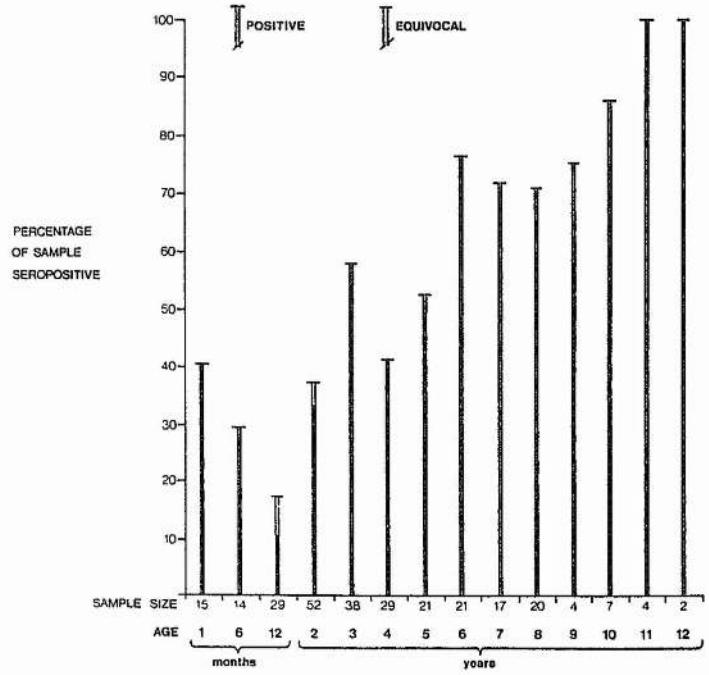

Fig. 2. Age distribution of anti-FRKV, detected by CIE, in 273 cattle

Commercially Available Sera

Thirty-five different batches of cattle serum pools, marketed for tissue culture work, were obtained from several commercial sources and tested for anti-FRKV by CIE. The results are shown in Table 5. Overall, 69 per cent of these pooled sera contained anti-FRKV, but whereas all of the newborn calf sera contained anti-FRKV, only 59 per cent of the foetal calf sera were definitely positive and 22 per cent did not have detectable levels of anti-FRKV when tested by CIE.

Table 5. *Results of testing 35 commercially produced cattle serum pools for presence of anti-FRKV by CIE*

Serum	Positive	Equivocal	Negative	Total
Foetal calf	16 (59%)	5 (19)%	6 (22%)	27
Newborn calf	7 (100%)	—	—	7
Donor calf	1 (100%)	—	—	1
Totals	24 (69%)	5 (14%)	6 (17%)	35

Colostrum Deprived Calves

Thirteen sets of mothers and calves which were not included in the detailed analysis above (b) were tested for anti-FRKV. The calves were bled immediately after birth and were not permitted to suckle, in order to prevent transfer of maternal antibody in the colostrum. Only two sets of mother and calf, CD2 (CIE titres: neat and 1/16 respectively) and CD10 (CIE titres: both 1/8) had detectable levels of anti-FRKV in their serum. Serum from one additional mother, CD12, gave rise to a definite, but very faint precipitin line in CIE, which was not typical of the specific reaction. Colostrum was also available for 12 of the sets and these were tested for anti-FRKV. (Unfortunately colostrum was not available from CD10.) It was found that anti-FRKV was not only present in the colostrum of the seropositive mother, CD2 (CIE titre 1/32), but also in those of CD1, CD7, CD8, and CD11 (CIE titres: 1/8, 1/2, 1/4 and 1/8 respectively). Colostrum from CD3 reacted very weakly when tested undiluted. In mother CD2 the titre of anti-FRKV in the colostrum was approximately 32-fold higher than in her serum.

Double Diffusion Tests on Animal Sera

In order to investigate serological identity between naturally occurring anti-FRKV and that prepared in rabbits the following specimens were selected for GD tests as a representative sample of those that were reactive by CIE: two batches of commercial newborn calf serum, 2 sera from colostrum deprived newborn calves, 3 sera from adult cattle, 2 bovine colostrum samples, 1 goat and 1 pig serum.

The results fell into 3 categories. Ten samples gave a line of identity with the rabbit antiserum reaction, but these could be placed in 2 further sub-divisions: those which gave only a line of identity, of which there were 6 (5 bovine and 1 goat) and those samples (2 colostra and 2 bovine sera) which gave rise to other

lines in addition to the one continuous line which signifies identity: one gave rise to a spur, another to a second line of identity and two did not show identity of reaction. Finally, one sample, the pig serum failed to react with the FRKV antigen but a strong reaction occurred between the pig serum and both the pre-immune and immune rabbit sera which were in adjacent wells. The adjacent rabbit antiserum did continue to precipitate the FRKV antigen.

Discussion

Polyomaviruses are considered to be essentially host specific in their *in vitro* growth characteristics (5). The results presented here and elsewhere show that FRKV and STMV do not grow very readily in the primate kidney cultures in which they were discovered. FRKV was detected only after extended incubation of the FRhK-4 and FRhK-6 cell cultures (10). Similarly, SHAH *et al.* (11) were unable to find specific STMV IF until at least 8 weeks after initially preparing the kidney cultures, when it was demonstrated in tertiary cultures. Calf kidney cultures, however, proved to support replication of FRKV very well. Indirect immunofluorescence demonstrated that a much greater proportion of CK cells, 75 per cent, became infected with FRKV (10) than was the case for the FRhK-4 and stump-tailed macaque kidney cultures, in which only 5—10 per cent had specific nuclear fluorescence (7, 9). The FRKV inoculated CK cultures did not require lengthy incubation to produce an adequate antigen for CIE. This antigen may not have needed purification as it was little affected by the non-specific reactivity seen in the crude FRhK-4 derived antigen which required purification and concentration before use.

The serological results obtained by IEM and IF (10) and those of CIE and GD reported here indicate that, at very least, STMV and FRKV are closely related antigenically. As STMV was not available to carry out two-way matching, the possibility does exist of a one-way cross reaction, but none of the other polyomaviruses seems to possess such an inter-typic antigenic relationship. This leads us to conclude that STMV and FRKV, and consequently HD, are one and the same polyomavirus.

Because FRKV was isolated from rhesus monkey tissues it might have been concluded that the natural host of this virus is the rhesus monkey, in much the same way that STMV has been related to stump-tailed macaques. The failure to demonstrate anti-FRKV in rhesus monkeys parallels the failure to find anti-STMV in stump-tailed macaques in which this phenomenon has been explained using a hypothesis of vertical transmission and tolerance in the infected offspring (11). In this respect STMV, and therefore FRKV, would stand alone amongst the known polyomaviruses (Table 1). All kidney cultures prepared from 27 adult and foetal stump-tailed macaques yielded STMV (11) and so it has been postulated that STMV carriage is very common, if not universal in the stump-tailed macaque population (12), but the presence of STMV or its antigens has never been demonstrated *in vivo*. The hypothesis, however, is unlikely because stump-tailed macaques have been challenged with STMV and shown to mount an immune response indicating that these animals had not been rendered tolerant (12).

Evidence of natural exposure to FRKV was found almost entirely in cattle, of which a large proportion were found to be seropositive. FRKV therefore seems commonly to infect cattle, though nothing is known which connects the virus to any particular disease. The finding of anti-FRKV in 1/100 pigs or 1/20 goats might be explained if they had been in close proximity to cattle or to their body tissues or fluids. The relationship between age and seropositivity suggests that a proportion of newborn calves receive passive anti-FRKV from colostrum which becomes undetectable after several months, and that they subsequently become actively immune due to infection by horizontal transmission of FRKV. An alternative explanation is that the increase in anti-FRKV prevalence with increase in age is due to reactivation of a latent FRKV infection in at least some of the cows. This could then boost anti-FRKV from undetectable to detectable levels by CIE. Because the half-life of bovine IgG is about 3 weeks it is improbable that CIE is sufficiently sensitive to detect anti-FRKV which has been acquired passively, during the first day after birth, for a period of 9—12 months.

The detection of anti-FRKV in the colostrum of cows which were seronegative is not an anomaly; colostrum is known to contain several times the concentration of IgG found in serum and may therefore be positive when the serum appears to be negative, thus offering a more sensitive means of demonstrating previous exposure to the virus in pregnant cows near term. The high level of colostral IgG is attained by transfer from, and consequent depletion of, serum IgG in the pregnant cow. The maternal serum IgG levels return to normal within 24 hours after calving. A detectable CIE reaction from the serum of these cows may thus indicate a recent antigenic stimulus.

Reactivation during pregnancy has been demonstrated in the case of the human polyomaviruses, BK and JC (3), and this may also occur with bovine polyomavirus in some pregnant cattle. Because transfer of maternal antibody through the bovine placenta does not occur, the presence of anti-FRKV in the colostrum deprived calves may be interpreted as evidence that an intra-uterine infection with FRKV has occurred. This conclusion is supported by the observation that only the mothers of the 2 seropositive calves were seropositive although other mothers had anti-FRKV in their colostrum indicating previous experience of FRKV. The demonstration of serum anti-FRKV may indicate a recent infection or reactivation in these 2 cows, presumably during pregnancy, and consequent infection of their foetuses.

The finding of anti-FRKV in a substantial proportion of FBS batches has similar significance, indicating that some of the donations added to the pool must have been from foetuses which had been infected with FRKV. Because of the method of bleeding the foetuses (from the heart) it is most unlikely that contamination of the pool with sufficient seropositive maternal blood to cause positive results in CIE could occur.

If intra-uterine infections do occur then it is likely that some of the batches of FBS may have contained one or more donations carrying infectious FRKV. Although in some cases there may be sufficient anti-FRKV in the serum pool to cause neutralisation, some FBS pools do not contain detectable levels of anti-FRKV and these may occasionally contain infectious FRKV. Investigation of this may prove extremely difficult because there could be too few virus particles

present to be detectable by direct means and because most cell cultures have been, and are, grown on medium containing FBS and therefore semi-permissive cells may already be infected with FRKV.

Attempts to show complete identity by GD between the FRKV antiserum raised by rabbit immunisation and the naturally occurring antibodies detected by CIE in farm animals were in some cases difficult to interpret because more than one precipitin line was present. This might be due to a difference in response between immunisation and natural infection. The natural response seems to be more complex and the additional precipitin lines in GD may be due to more than one class or sub-class of immunoglobulin. The results do indicate in some cases at least partial and in others complete identity of the antigenic stimulus given by the polyomavirus in cattle and that given by FRKV, and by inference STMV, in rabbits.

In 1980, Coackley *et al.* (1) reported the detection of a polyomavirus, which they called WRSV, in tissue cultures prepared from the kidneys of a healthy newborn colostrum deprived calf. The presence of the virus was discovered when eosinophilic intra-nuclear inclusions were observed in CK coverslip cultures. The virus was readily passaged in CK cells, but replication was not detected in several other cell culture systems. WRSV failed to agglutinate red blood cells from chickens, sheep, guinea pigs and man. We are planning to undertake serological tests with WRSV in order to clarify the relationship between STMV, FRKV and WRSV. It seems likely that this probable bovine polyomavirus, WRSV, is a naturally occurring strain of these viruses.

The evidence so far points to the conclusion that FRKV is very similar to the virus designated STMV. Serological data lead us to believe that cattle, not primates, are the natural hosts of FRKV and consequently STMV. On the basis of studies of colostrum deprived calves and FBS samples we would, in addition to horizontal transmission, propose that *in utero* transmission of FRKV due to either reactivation or primary infection occurs in a significant proportion of pregnant cattle. This would explain how primate tissue culture lines could become infected with a bovine polyomavirus since some batches of FBS may contain infectious FRKV. The infection may be non-cytopathic and therefore go unrecognised unless positive measures are taken to detect it.

Several questions remain unanswered: how widespread is the contamination of laboratory cell lines with this bovine polyomavirus, especially those being used in the field of vaccine development? How significant would the finding of this polyomavirus be in such cell lines? Is FRKV capable of infecting man and if so what might be the outcome? Although we have reported its ability to replicate in human embryonic kidney cells (7, 10), we have so far been unable to find evidence of anti-FRKV in human sera (Table 4).

Addendum

Subsequent to the preparation of this paper we have been able to carry out some preliminary serological tests on the bovine polyomavirus WRSV, kindly provided by Mr. W. Coackley, Animal Health Laboratory, S. Perth, Western

Australia. We have demonstrated that WRSV reacts by CIE with 2 rabbit antisera to STMV (75—176 and 81—239), the rabbit anti-FRKV and newborn calf serum, NBS 22/6. WRSV also gave specific nuclear fluorescence with rabbit anti-STMV (75—176) in IF. WRSV does therefore seem to be antigenically very similar to STMV and FRKV.

Acknowledgments

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Characterisation of a Polyomavirus in Two Foetal Rhesus Monkey Kidney Cell Lines Used for the Growth of Hepatitis A Virus

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With 14 Figures

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Summary

Electron microscopy and prolonged incubation of cell cultures have revealed the presence of a papovavirus in two foetal rhesus monkey kidney cell lines, FRhK-4 and FRhK-6, which are used to grow hepatitis A virus. The papovavirus, designated FRKV, was present in culture fluids from both cell lines and in thin sections of FRhK-4 cells. The size of the virus, 47 nm, places FRKV within the Polyomavirus genus. FRKV has been grown in primary human embryo kidney and calf kidney cell cultures. Haemagglutinin has not been demonstrated.

Preliminary investigations indicate that FRKV is antigenically identical to or closely related to stump-tailed macaque virus and is not one of the primate polyomaviruses SV40, SA12, BK or JC. FRKV reacts with antibody that occurs in pools of foetal and newborn bovine sera suggesting that the virus might be of bovine origin.

Introduction

Continuous cell lines and cell cultures derived from kidney tissues from various animals, especially monkeys, have been reported to be infected with viruses belonging to the Polyomavirus genus of the Papovaviridae (4, 8, 15, 18, 25, 28, 32). One of these viruses, simian virus 40 (SV40), caused much concern when it was discovered in virus seed stocks and batches of polio vaccine widely used in man between the years 1955 and 1963 (26, 28).

SV40 and two other polyomaviruses, simian agent 12 (SA12) and stump-tailed macaque virus (STMV) were found originally in kidney cell cultures from rhesus, African green and stump-tailed macaque monkeys respectively (15, 24, 28). These three polyomaviruses have been investigated and shown to be immunologically distinct and to have different genomes (17). More recently a cell line (Vero) derived from kidney tissue of an African green monkey has been shown to be chronically infected with polyomavirus HD, which is identical to STMV (11, 32). Polyomaviruses have also been found in non-primate cell cultures: in a pig kidney cell line PK-15 (18) and in cells prepared from the kidneys of a newborn calf (4). Neither of these last two viruses has been fully characterised.

In 1973 Wallace and her colleagues established several cell lines from foetal rhesus monkey tissues including kidney (34). Two of these lines, FRhK-6 and FRhK-4, have been used to grow hepatitis A virus (HAV) (7, 23). An experimental live attenuated HAV vaccine is being studied in chimpanzees (22) and trials are planned for human volunteers (10). The seed HAV used in the preparation of this vaccine was passaged from 5 to 25 times in FRhK-6 cells before being adapted to grow in human diploid lung fibroblast cell cultures.

We report here the detection, isolation and preliminary identification of a polyomavirus present in FRhK-4 and FRhK-6 cultures. The FRhK-4 cell line was originally obtained in order to investigate its suitability for producing HAV antigen for use as a diagnostic reagent. Polyomavirus particles were detected by electron microscopy (EM) when fluids from HAV-inoculated and uninoculated FRhK-4 cultures were examined after prolonged incubation. To determine whether or not this virus is a recent contaminant, lower passage FRhK-4 and FRhK-6 cells were obtained from the Bureau of Biologics, Maryland, which holds the original cell stocks. Polyomavirus particles were also detected in these cultures.

Materials and Methods

Cell Culture and Propagation of Papovavirus

FRhK-4 cultures, pass 76, were obtained from Dr. B. Flehmig, Tübingen University, Germany (FRhK-4). FRhK-4, pass 21 (FRhK-4/B), and FRhK-6, pass 5, cultures were supplied by Dr. J. C. Petricciani, Bureau of Biologics, Bethesda, U.S.A. The cells were grown at 37° C on Eagle's minimum essential medium (MEM) containing 6 or 10 per cent foetal bovine serum (FBS) (Flow Laboratories), and maintained at 33° C on MEM with 2 per cent FBS which was changed at approximately weekly intervals. FRhK-4/B and FRhK-6 cells were manipulated in a separate laboratory from FRhK-4 cells, with their own culture media, to reduce the risk of contamination with the FRhK-4-derived papovavirus.

FRhK-4 cells were passaged twice at approximately monthly intervals before inoculation with HAV, which is described in detail elsewhere (19). After 42 days incubation at 37° C culture fluids from both HAV-inoculated and control uninoculated

cells were examined by EM and found to contain papovavirus particles. To further investigate this papovavirus, an uninoculated stock culture of FRhK-4 cells was maintained at 33° C for 6 to 8 weeks before subdivision when several extra cultures were made. FRhK-4, FRhK-4/B and FRhK-6 cells were maintained at 37° C on either MEM with 2 per cent FBS or Iscove's modification of Dulbecco's MEM, without serum. The culture fluids, cells which spontaneously detached from the cell sheet, and monolayer cells were used as sources of papovavirus for all investigations.

Antisera

Three STMV antisera, two prepared in rabbits, and one in a rhesus monkey, were kindly provided by Dr. K. V. Shah (Johns Hopkins University, Baltimore, U.S.A.) who also supplied rabbit antisera against SA 12 and SV 40. Mr. J. Hill (Pfizer Ltd., Kent, U.K.) kindly provided the SV 40 calf antiserum. Rabbit and guinea pig antisera raised against the human polyomaviruses BK and JC, and a rabbit serum raised against FRKV were prepared in our laboratory. In addition, two pools of newborn bovine sera supplied for cell culture (Flow Laboratories) were examined.

Electron Microscopy (EM) Negative Stain

Monolayer cells were washed with phosphate buffered saline (PBS), scraped off the flasks into PBS then centrifuged at $1500 \times g$ for 10 minutes. For the examination of cells that had become detached from the monolayers during incubation, culture fluids (5mls) were similarly centrifuged. High speed pellets were prepared by centrifuging 3 or 5 ml volumes of culture fluids or clarified culture fluids at $48{,}000 \times g$ for 1 hour or at $150{,}000 \times g$ for 2 hours. All pellets were resuspended in small volumes of distilled water (approximately 20 µl), applied to formvar/carbon-coated grids and negatively stained with 3 per cent phosphotungstic acid, pH 6.3. For extraction of virus, detached cells from 5—25 ml volumes of culture fluids were washed with PBS then centrifuged at $1500 \times g$ for 10 minutes. The pellets were resuspended in 20—40 µl distilled water, treated in an ultrasonic bath for 2 minutes, recentrifuged, and the supernatants negatively stained. Attempts to purify the virus extracted from detached cells by treatment with nonidet P 40 for 30 minutes at 37° C or by homogenisation with fluorocarbon followed by centrifugation were also made.

Measurement of FRhK-Derived Papovavirus

The microscope magnification was calibrated using crystalline catalase (36). Suspensions of catalase, FRhK-4-derived papovavirus and human papillomavirus were negatively stained as described above. The diameters of 100 morphologically characteristic virions from both preparations were measured. Damaged and aberrant forms were not included and almost all of the particles measured appeared "full" (5).

EM Thin Sections

Monolayers of HAV-inoculated and control uninoculated FRhK-4 cells (pass 81), that had been incubated for 55 days, were fixed *in situ* in cold 2.5 per cent glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The cells were scraped into fresh fixative and centrifuged at $10{,}000 \times g$ for 2 minutes in an Eppendorf centrifuge. The pellets were washed, post-fixed in osmium tetroxide and dehydrated (31), then embedded in Epon as described by Luft in 1961 (14). Thin sections were stained with alcoholic uranyl acetate followed by lead citrate.

Virus Isolation

Culture fluid from FRhK-4 cells (pass 78) incubated for 42 days was inoculated into primary human embryo kidney cell cultures (HEK). The cells were maintained on Eagle's MEM and 2 per cent NBS and incubated at 39° C for 1 month. The medium was

changed three times a week. Subcultures were made from the inoculated HEK cultures, which were showing cytopathic changes, either directly from the medium or from infected cells which had been frozen and thawed three times and ultrasonicated to release virus particles. FRKV and HEK-adapted FRKV were also inoculated into secondary cultures of calf kidney (CK) cells.

Haemagglutination

Suspensions of FRKV were treated with either nonidet P 40 (BDH Chemicals Ltd.) for 30 minutes at 37° C or were incubated overnight with neuraminidase and then inactivated at 56° C for 30 minutes. Treated virus preparations were warmed at 37° C for 30 minutes before testing for the ability to agglutinate 0.5 per cent human 0 cells at 4° C and pH 6.3 and 0.5 per cent bovine red cells at 4° C and 37° C and pH 6.3. Untreated virus was also tested for haemagglutination with 0.5 per cent suspensions of erythrocytes from chicken, sheep, rhesus and baboon monkeys and man at 4°, 20° and 37° C and pH 7.

Immunoelectron Microscopy (IEM)

All preparations of FRKV for IEM were from uninoculated FRhK cultures: suspensions of virus extracted from spontaneously detached cells, or free virus from fluids from cultures maintained on serum-free medium were used. SV 40 and SA 12 were grown in Vero cells, and the appearance of the virus in all preparations was examined by EM before use. Antigens and sera were diluted in PBS. Specific antisera against SV 40 and SA 12 were used at the dilutions that resulted in dense antibody coating of their homologous viruses. Other antisera were known to react positively in IEM tests (Table 1). Equal volumes (5 µl) of antigens and sera were mixed on Parafilm and maintained in a moist chamber for 1 hour at room temperature. The mixture was applied to a formvar/carbon-coated grid then negatively stained with 3 per cent phosphotungstic acid.

Immunofluorescence

FRKV antigen for use in immunofluorescence tests was prepared either directly from FRhK-4 cells or from CK cells inoculated with FRKV. The cells were grown as monolayers on coverslips in multiwell chambers and incubated at 37° C in a CO_2 incubator. Between 6 and 19 days after seeding (FRhK-4) or post infection (CK) the coverslip cultures were fixed in acetone at room temperature for 20 minutes and stored at —30° C until required. FRKV-infected cells and control uninoculated CK cells were examined with type specific sera in an indirect method using fluorescein-labelled antibody against rabbit and human immunoglobulins (Wellcome Reagents Ltd.).

Results

EM Negative Stain of FRhK Cultures After Prolonged Incubation

Papovavirus particles were first detected by EM in culture fluids from both HAV-inoculated and uninoculated FRhK-4 cells (pass 78) after

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Figs. 1 to 5. Negatively stained preparations of foetal rhesus monkey kidney cell-derived polyomavirus, FRKV, × 200,000

Figs. 1 to 3. FRKV extracted from FRhK-4 or FRhK-6 cells that had become detached from the monolayers during culture. Medium contained 2 per cent foetal bovine serum

Fig. 1. A group of polyomavirions, 3 of which are associated with membrane. FRhK-4 culture after 99 days incubation

Fig. 2. Polyomavirions and part of a polyomavirus filament. Arrows indicate 2 small, featureless, electron-opaque particles. FRhK-6 culture (pass 7)

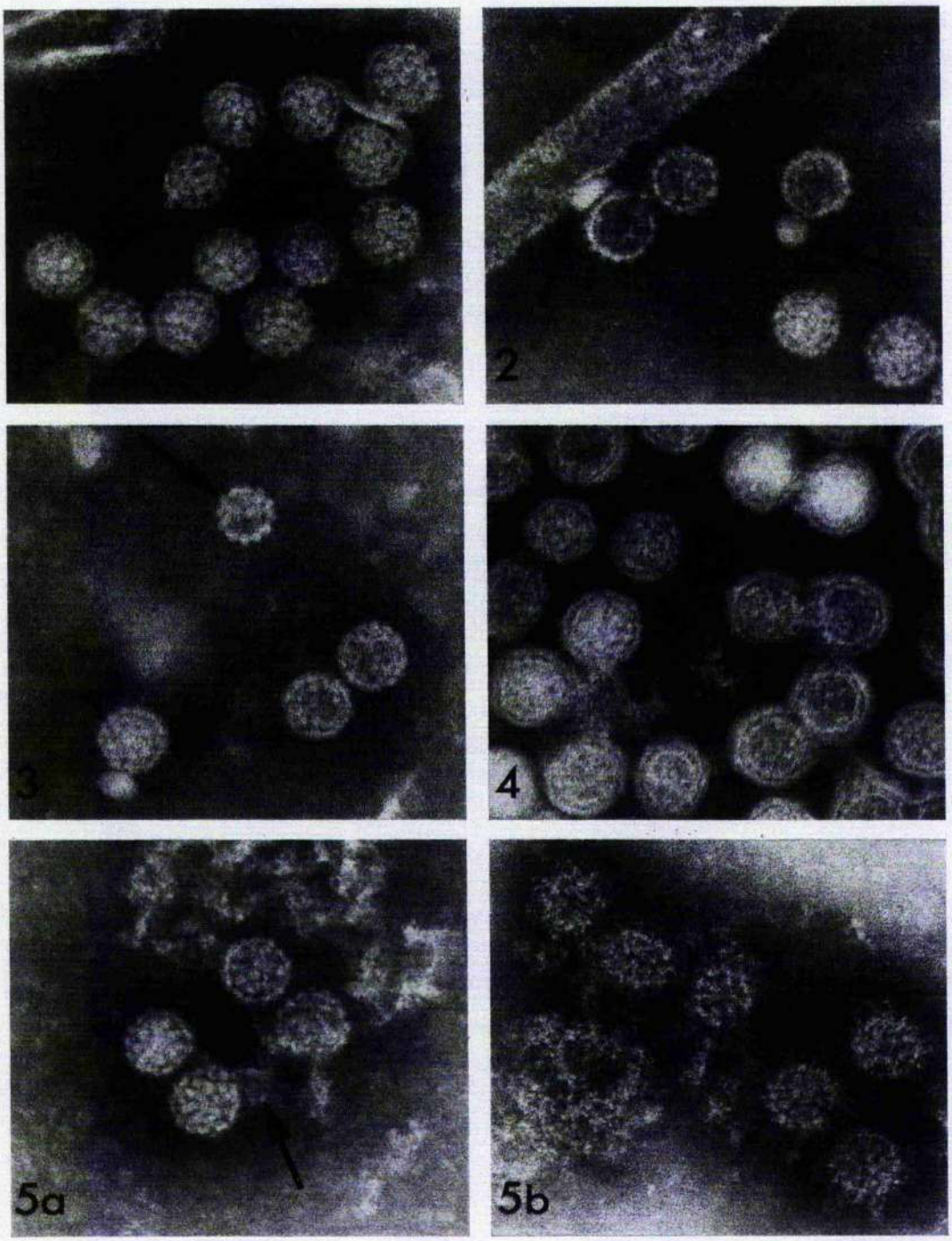


Fig. 3. Polyomavirions and a "mini" spherical polyomavirus particle (arrow). FRhK-4 culture

Figs. 4 and 5. High speed pellets of culture fluids from HEK cells 19 and 28 days after inoculation with FRKV. Medium contained 2 per cent newborn bovine serum, 17/8

Fig. 4. Membrane-bound polyomavirions

Fig. 5a. Amorphous substance (arrow) is associated with polyomavirions

Fig. 5b. Polyomavirions are surrounded by a dense coat of antibody

42 days incubation. Such particles were observed in larger numbers when uninoculated cultures were maintained for longer periods without subdivision (Fig. 1). Papovavirions could not be found in culture fluids examined from FRhK-4 cells that were repeatedly subdivided at 21 day intervals, nor from other cell lines maintained concurrently in the same laboratory.

Virus particles were not detected in either cell line obtained from the U.S.A. (FRhK-4/B or FRhK-6) when examined after 8 weeks in culture. However after 5 months, the presence of papovavirus was demonstrated in FRhK-4/B (pass 25) and in FRhK-6 (pass 7) (Fig. 2) approximately 5 weeks after the last subculture.

Moderate to large numbers of papovaviruses were seen in preparations of cells that had become detached from the monolayers during culture (Figs. 1 to 3), but virus particles were rarely found in negatively stained preparations of monolayer FRhK-4 cells. In all preparations, FRhK-derived virus particles occurred singly and in aggregates. There were small numbers of damaged particles, papovavirus filaments (Fig. 2), and "mini" spherical papovaviruses approximately 38 nm in diameter (Fig. 3). Small, round or oval, featureless, electron-opaque particles of varying size (Figs. 2 and 3) or fragments of cell membrane (Fig. 1) were frequently associated with the virus, and occasionally single or clustered virus particles were surrounded by membrane (Fig. 4). In addition, variable amounts of amorphous substance that did not resemble antibody (Fig. 5a) were attached to some of the virions and occasionally appeared to be holding the particles together in aggregates. Treatment of such samples with nonidet P40 or fluorocarbon considerably decreased the numbers of virus particles present without improving their appearance, and their use for purification of antigen for IEM was considered impractical.

High speed pellets of culture fluids that had not been clarified, comprising virus in detached cells and free virus, contained a mixture of "clean" virus particles like those in Figs. 1 to 3 and 5a, and papovaviruses coated with a substance morphologically indistinguishable from antibody (Fig. 5b). Small numbers of virus particles, almost all of which were antibody-coated, were observed in high speed pellets from clarified culture fluids. Antibody-coated papovaviruses were found in preparations from cultures maintained with medium containing 2 per cent foetal bovine serum (FBS) and the particles were more densely coated with antibody when the medium contained 10 per cent FBS or 2 per cent newborn bovine serum (NBS 17/8) (Fig. 5b). Moderate to large numbers of "clean" papovavirions were observed in high speed pellets of culture fluids from FRhK-4 and FRhK-6 cells grown on Iscove's serum-free medium; antibody-coated particles could not be found in such preparations.

Measurement of FRhK-Derived Papovavirus

Most of the FRhK-derived papovavirus particles observed were morphologically characteristic (Figs. 1 to 3 and 5a). The mean diameter of 100 such particles was 46.8 nm (range 44—50 nm), that of 100 human papillomavirions was 57.1 nm (range 53—60 nm).

EM Thin Sections of FRhK-4 Cells

Polyomavirus particles (Figs. 6 to 10) were observed in 5 to 10 per cent of the monolayer cells examined in both HAV-inoculated and uninoculated FRhK-4 cultures, incubated for 55 days. Approximately equal numbers of morphologically undamaged and degenerate cells contained polyomavirions most of which were found within the nuclei (Figs. 6 and 7). Small numbers of particles were usually scattered throughout the nucleoplasm, whereas larger numbers of virions were frequently randomly organised in loose groups. One paracrystalline array of polyomavirions was observed within the nucleus of a degenerate cell (Fig. 8), and polyomavirus filaments were occasionally seen (Fig. 9). In addition to intranuclear virus, a few cells contained polyomavirus particles that were either free within the cytoplasm or closely associated with cytoplasmic membranes (Fig. 10).

Virus Isolation and Haemagglutination

Cytopathic changes first appeared in HEK cell cultures 21 days after inoculation with culture fluid from FRhK-4 cells. The cytopathic effect (CPE) consisted of early cytoplasmic vacuolation followed by granular rounding of cells which detached from the cell sheet. Highly refractile round cells were also observed. At this time polyomavirus particles were detected in the culture fluids (Figs. 4 and 5) and monolayer cells by EM. On passage the interval before appearance of the CPE was shortened to 9 days.

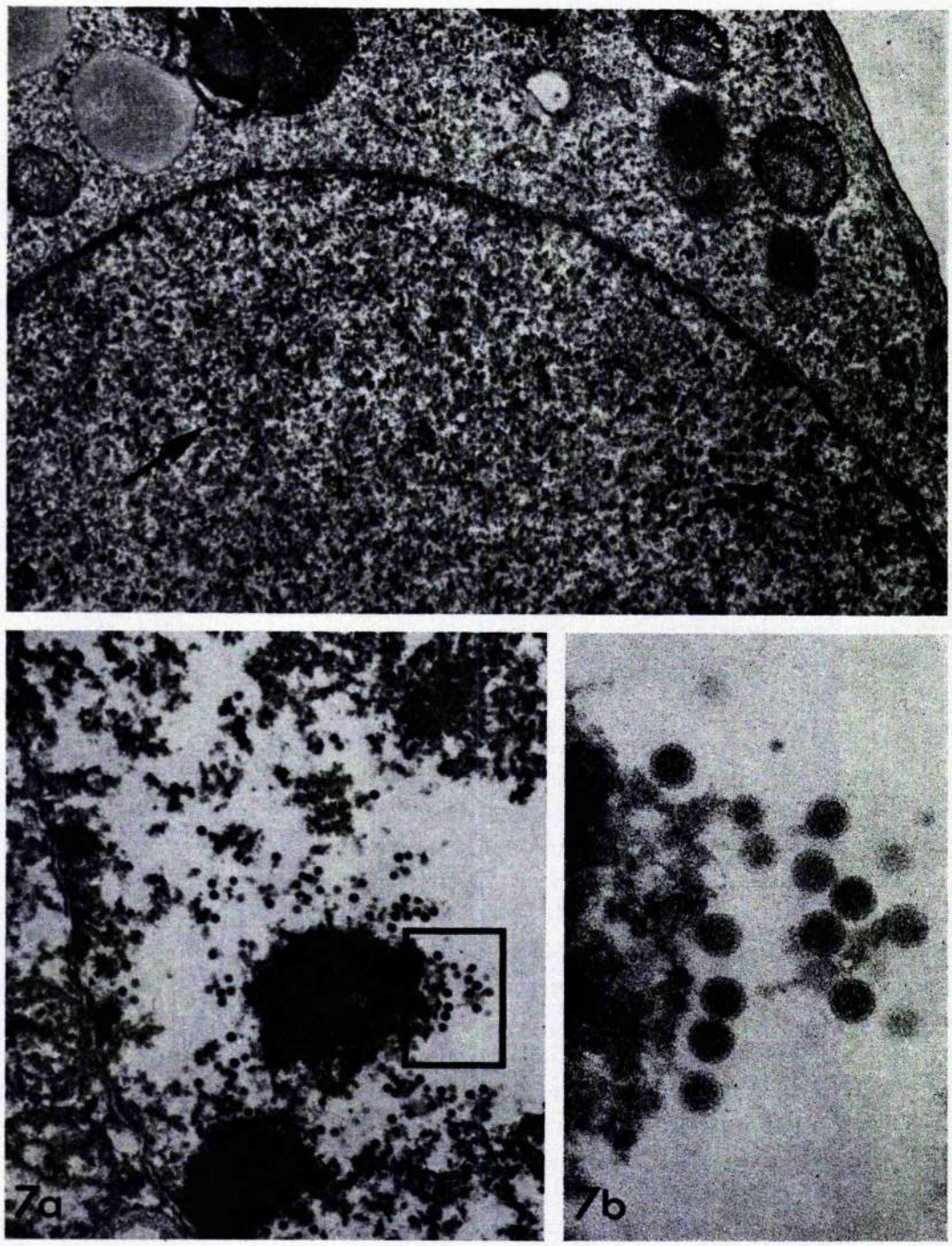
Both FRKV obtained directly from FRhK-4 cells and the HEK-adapted FRKV grew readily when inoculated into secondary calf kidney cells with an incubation period of about 9 days. Early cytoplasmic vacuolation was extensive in these cells followed by granular degeneration, and large numbers of polyomavirions were present in culture fluids.

Haemagglutination was not observed when FRKV was mixed with human 0, chicken, bovine, sheep, rhesus monkey and baboon monkey erythrocytes.

Identification of FRhK-Derived Polyomavirus FRKV

Immunoelectron Microscopy (IEM)

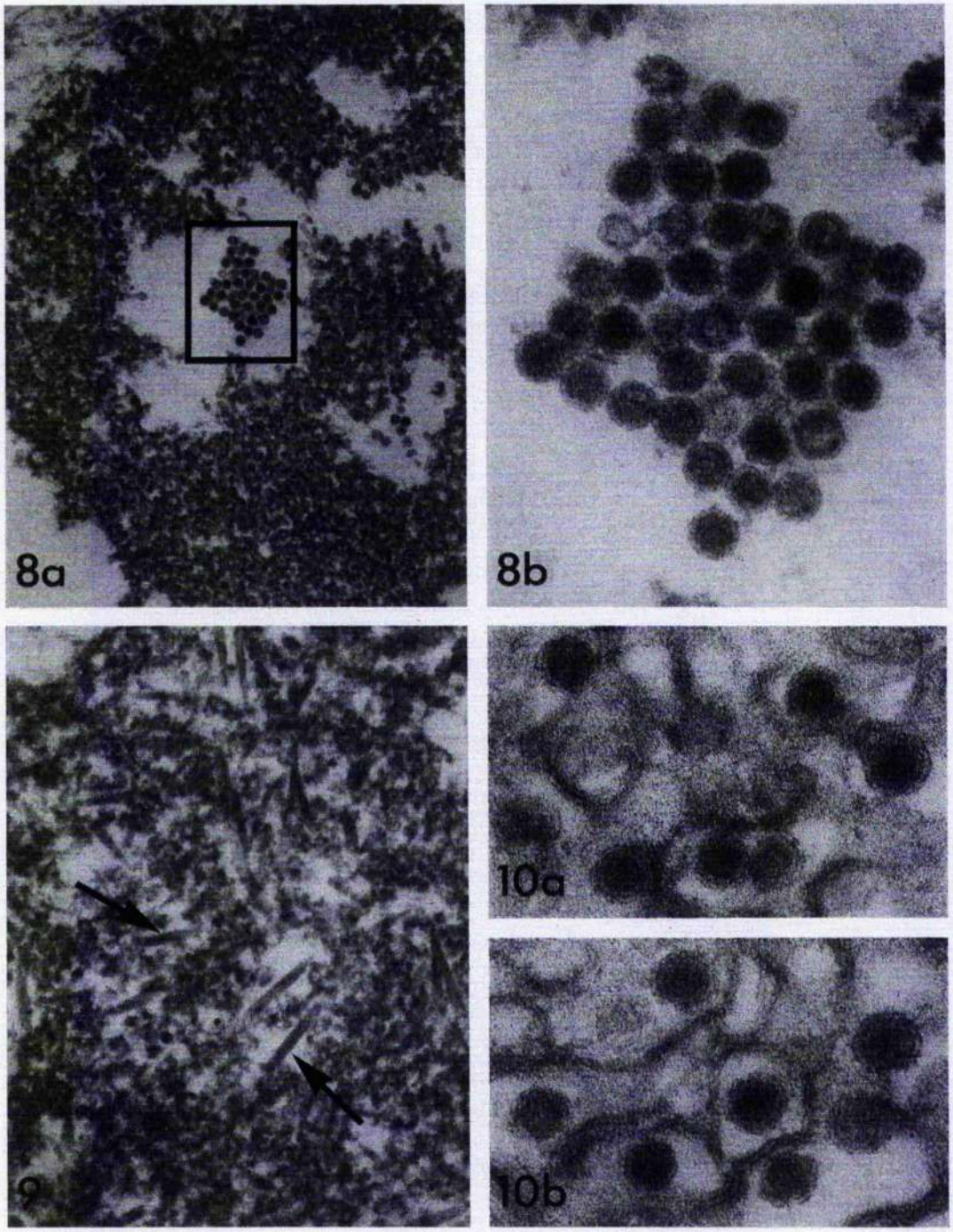
Because free virus from FRhK culture fluids containing bovine sera was antibody-coated, only virus from detached cells or from cultures maintained



Figs. 6 and 7. Thin sections of FRhK-4 (pass 81) monolayer cells 55 days after inoculation with hepatitis A virus

Fig. 6. Part of a morphologically undamaged cell. Arrows indicate intranuclear polyomavirions randomly organised in loose groups $\times 30{,}000$

Fig. 7. Part of a degenerate cell *a* $\times 30{,}000$ and *b* polyomavirions in the area of nucleus outlined $\times 150{,}000$



Figs. 8 to 10. Thin sections of control, uninoculated FRhK-4 (pass 81) monolayer cells after 55 days incubation

Fig. 8. Polyomavirions in paracrystalline array are surrounded by the chromatin of a degenerate nucleus *a* ×30,000 and *b* the area outlined ×150,000

Fig. 9. Arrows indicate filamentous polyomavirus particles ×50,000

Fig. 10. Cytoplasmic polyomavirions closely associated with membranes: some particles are loosely surrounded by membrane, others appear to be completely enveloped ×150,000

on Iscove's medium was used. Each batch of antigen prepared was examined before use to confirm that particles were not coated with antibody; the virions illustrated in Figs. 1 to 3 and 5a are representative of those in virus suspensions used for IEM. Groups of papovaviruses occurred in all preparations, despite ultrasonication, thus the presence of aggregates of virus after incubation with test sera could not be interpreted unequivocally as the result of interaction between virus and antibody. IEM results were therefore only scored positive if virus particles were surrounded by antibody molecules (Figs. 11 to 14). Since the amorphous substance attached to some virus particles made detection of sparse antibody coating difficult, no attempt was made to further dilute sera in order to quantitate the amount of antibody present.

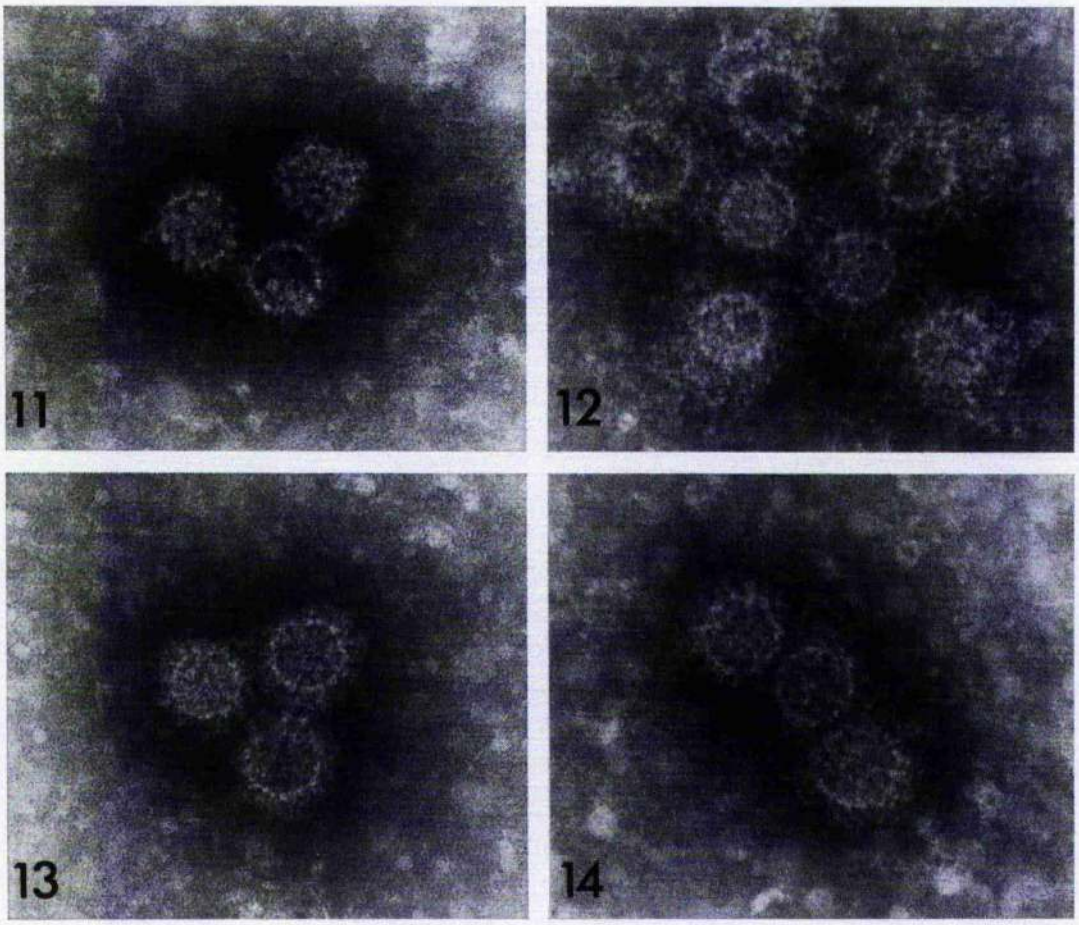
The results of IEM tests of FRKV with specific polyomavirus antisera and newborn bovine sera are given in Table 1. Antibody coating of FRhK-4-derived virus was not observed with antisera against BKV, JCV, SV40, SA12 or the monkey STMV antiserum. However, polyomavirus particles from both FRhK-4 and FRhK-6 cultures were densely coated with antibody after incubation with the rabbit STMV antisera (Figs. 11 and 12) and with both batches of newborn bovine sera (Figs. 13 and 14). When rabbit FRKV antiserum was tested with FRhK-4-derived virus equivocal results were obtained: there was an increase in the numbers and sizes of aggregates of virus, and small amounts of antibody were present on the surface of the particles, but dense antibody coating was not observed.

Table 1. *Immunoelectron microscopy of FRKV with specific polyomavirus antisera and newborn bovine sera*

Serum	Dilution	Homologous virus	FRKV from FRhK-4 cultures	FRKV from FRhK-6 cultures
BKV guinea pig	1/15	+[a]	—	nt
JCV guinea pig	1/15	+[a]	—	nt
JCV human	1/10	+[a]	—	nt
SV40 calf	1/25	+	—	nt
SA12 rabbit	1/10	+	—	nt
STMV monkey (77/4286)	1/5		—	nt
STMV rabbit (75/176)	1/10		+	+
STMV rabbit (81/239)	1/10	+[b]	+	+
NBS calf (17/8)	1/10		+	+
NBS calf (22/6)	1/10		+	+

+ = Polyomavirus particles that are densely coated with antibody
— = Polyomavirus particles that are not coated with antibody
nt = not tested

[a] Dr. A. M. Field, personal communication
[b] Dr. K. V. Shah, personal communication



Figs. 11 to 14. Positive IEM reactions illustrating polyomavirus particles that are densely or very densely coated with antibody $\times 200{,}000$

Fig. 11. Polyomavirions from FRhK-4 culture + rabbit STMV antiserum 75/176, 1/5

Fig. 12. Polyomavirions from FRhK-4 culture + rabbit STMV antiserum 81/239, 1/5

Fig. 13. Polyomavirions from FRhK-4 culture + newborn bovine serum 17/8, 1/10

Fig. 14. Polyomavirions from FRhK-6 culture + newborn bovine serum 22/6, 1/10

Immunofluorescence

Initially immunofluorescence was used to detect the presence of antigen both in FRhK-4 and FRhK-4/B cells using an anti-FRKV rabbit serum. Characteristic polyomavirus fluorescent staining of nuclei with sparing of the nucleoli was detected in both cultures but more nuclei were reactive in FRhK-4 cells than in the FRhK-4/B cells. Usually the cytopathic changes in both cell cultures were minimal. In an occasional coverslip extensive spontaneous degeneration of the monolayer was observed which appeared to be specific and in these cultures the fluorescence was strongly positive.

FRhK-4 cells were used as antigen in subsequent tests. Approximately 10 per cent of the cells gave specific nuclear fluorescence. Antigens were

also prepared in secondary calf kidney cultures after FRKV had been shown to grow in these cells: about 75 per cent of the nuclei gave positive fluorescence. Positive fluorescent staining of nuclei was observed when FRKV antigens were reacted with three different STMV antisera, one prepared in a rhesus monkey and two in rabbits. The rhesus monkey STMV antiserum was known not to contain SV40 antibody (25). Specific nuclear fluorescence was not detected when FRKV antigens were reacted with specific rabbit antisera to BKV, JCV, SV40 and SA12, or with human sera to BKV or JCV nor when uninoculated CK cells were examined.

Discussion

The detection of an unexpected adventitious virus during work with HAV in continuous lines of foetal rhesus kidney cells has raised the possibility of contaminated stocks of viruses grown in these cells. This virus, at present designated FRKV (foetal rhesus kidney virus), has the morphological appearance and intranuclear development characteristic of members of the Papovaviridae. The mean diameters of 57 nm for human papillomavirus and 47 nm for FRKV are comparable to those described respectively for papillomaviruses (5) and polyomaviruses (3, 4, 5, 6, 8, 12, 18, 25, 35) thus confirming that FRKV belongs to the Polyomavirus genus.

The presence of papovavirus filaments, "mini" spherical particles and membrane-associated virions in preparations of FRKV was not considered unusual. Filaments have been reported (1, 3, 16, 18), and also "mini" virus particles 38 nm and 22 nm in diameter (16). In addition, the association of polyomavirions with cell membranes has been seen in negatively stained preparations (4, 13, 16, 18, 25) and in thin sections (6, 9, 12, 13). The small, featureless particles attached to FRKV in some preparations (Figs. 2 and 3) were variable in size and shape and did not resemble the picornavirus-like particles reported in 1974 by Tischer and co-workers in a porcine kidney cell line that was also infected with a polyomavirus (29). Similar small, featureless particles have been illustrated in suspensions of SA12, STMV, SPV and WRSV (4, 12, 18, 25, 30) and are thought to be components of bovine sera (30). The fine amorphous substance we observed attached to some FRKV particles may be cellular in origin.

Polyomavirions have been detected in FRhK-4 cultures obtained from two different sources (FRhK-4 from Germany and FRhK-4/B from the U.S.A.) and in the FRhK-6 cell line. The possibility that the cells have become infected during culture in our laboratory cannot be discounted. However, these cell lines were among the 28 established by Wallace and her colleagues in 1973 (33, 34) who at that time reported cellular changes consistent with polyomavirus infection in 5 fibroblastic and 3 foetal rhesus kidney cell cultures. It was concluded by the authors that 27 of the 28 cell

lines, including FRhK-6, should not be used in the production of virus vaccines (34). Although we have shown that FRKV grows in primary human embryonic kidney cells, we do not know if the virus will grow in the human diploid cells, MRC-5 and Wl-38, in which FRhK-adapted HAV is passaged for vaccine production (22).

Immunoelectron microscopy has been effectively used to identify strains of polyomaviruses (1, 6, 21, 25, 30) and we have applied this technique to the study of FRKV. Our results indicate that the virus isolated from FRhK-4 and FRhK-6 cultures is the same as or very similar to STMV and this was supported by the findings with immunofluorescence. FRKV also has biological properties similar to STMV: cytoplasmic vacuolation is a prominent feature and is illustrated in our previous paper (20); also FRKV like STMV fails to agglutinate erythrocytes of various animal species. The attachment of amorphous substance to virions is characteristic of both FRKV and STMV (25).

STMV was first discovered by RANGAN and co-workers in 1974 (24) who reported that kidney cell cultures from 15 stump-tailed macaques *(Macaca speciosa)* all showed cytoplasmic vacuolation, which was often transient, after the cells had been passaged a few times. Subsequently STMV was identified as a new papovavirus by REISSIG and her colleagues in 1976 (25) and was grown in rhesus monkey kidney cells. STMV was thought to be unique amongst the papovaviruses in that the virus was always present in kidney cultures from stump-tailed macaques of all ages, and it has been suggested that STMV is normally congenitally transmitted (27). STMV, as well as being present in kidney cells from stump-tailed macaques, is now known to chronically infect Vero cells (11, 32).

Thus it seems that stump-tailed macaque kidney cultures, Vero cells and foetal rhesus monkey kidney cell lines have independently become infected with antigenically similar polyomaviruses. The antibody coating of free FRKV from high speed pellets of FRhK and FRKV-inoculated HEK culture fluids (Fig. 5b), which was indistinguishable from IgG antibody coating illustrated by ALMEIDA and WATERSON in 1969 (2), was observed when the medium contained foetal or newborn bovine serum. This finding, together with the dense antibody coating induced by the addition of either pool of newborn bovine serum to FRKV in IEM tests, led us to consider that the virus might be of bovine origin. The results of further investigation on the origin of FRKV are reported separately (20).

The presence of antibody in the medium could inhibit the growth of virus in cells that may already be infected making detection difficult. We suggest, therefore, that all cell cultures involved in virus vaccine production should not be used without first having been maintained in culture with minimal amounts of serum, or serum-free media, for at least 3 months, and specific evidence of polyomavirus infection sought but not found.

Acknowledgements

We are most grateful to Dr. N. G. Wrigley, National Institute for Medical Research, Mill Hill, for crystalline catalase, and Mr. J. R. Lukey, Central Veterinary Laboratory, Weybridge, for providing the primary calf kidney cell cultures. We also thank Dr. M. S. Pereira and Dr. A. M. Field for helpful discussions, Mrs. E.C. Paddon for assistance with photography and Miss B. Mandalia for typing the manuscript.

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THE DETECTION AND IDENTIFICATION BY ELECTRON MICROSCOPY OF A POLYOMAVIRUS (FRKV) CONTAMINATING FETAL RHESUS MONKEY KIDNEY CELL LINES USED TO GROW HEPATITIS A VIRUS

by

Joan Elizabeth Richmond

FIGURES

INDEX OF FIGURES

Figures 1 to 29 are electron micrographs of preparations negatively stained with phosphotungstic acid.

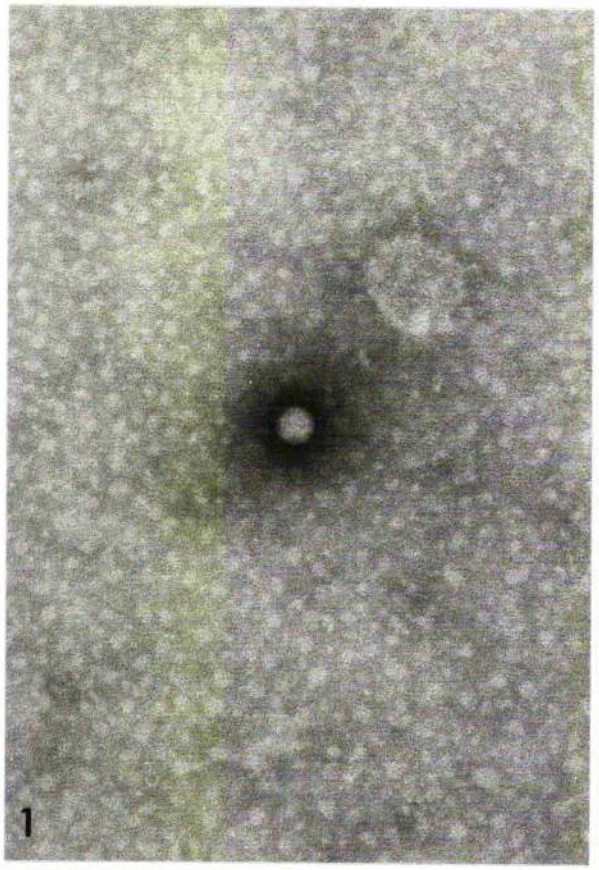
Figures 1 and 2 are of preparations of spontaneously detached cells from FRhK-4 culture 86 days after inoculation with HAV.

Fig 1. The characteristic appearance of negatively stained picornavirus group particles is illustrated by a single picornavirion, only very small numbers of which could be found. x 200,000.

Fig 2. IEM: HAV (grown in FRhK-4 cells) + purified anti-HAV IgG, 1/20. Four picornavirus particles are clumped by a dense coat of IgG and a plume or trail of electron-translucent substance can be seen. x 200,000.

Fig 3. IEM: HAV (MS-1 strain, marmoset faecal extract) + purified anti-HAV IgG, 1/40. Picornavirus particles, one of which has been penetrated by phosphotungstic acid, are surrounded and clumped by dense IgG. x 200,000.

Fig 4. Spontaneously detached cells from control, uninoculated FRhK-4 culture (pass 78) after incubation for 42 days; medium contained 2% fetal bovine serum. Five polyomavirions appear to be held together by fine strands, although antibody cannot be identified. x 200,000.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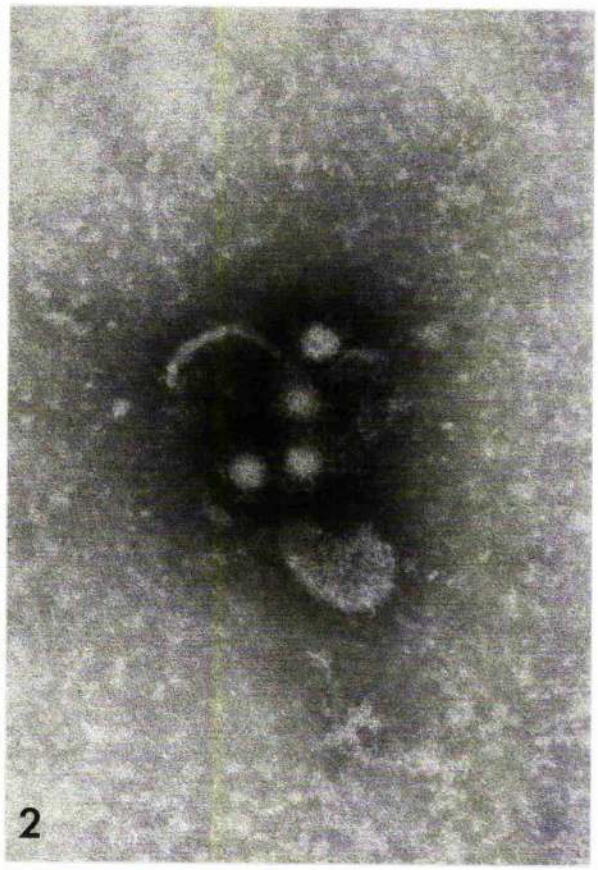
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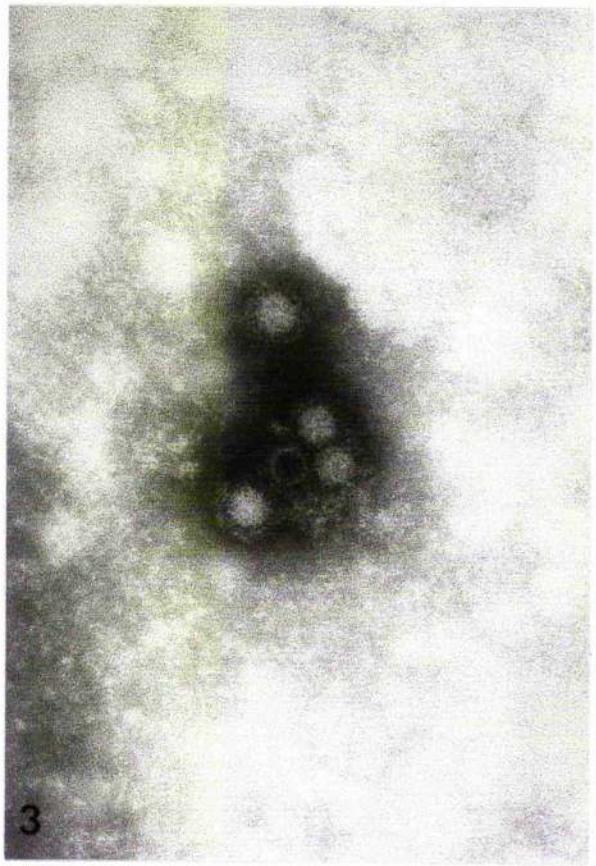
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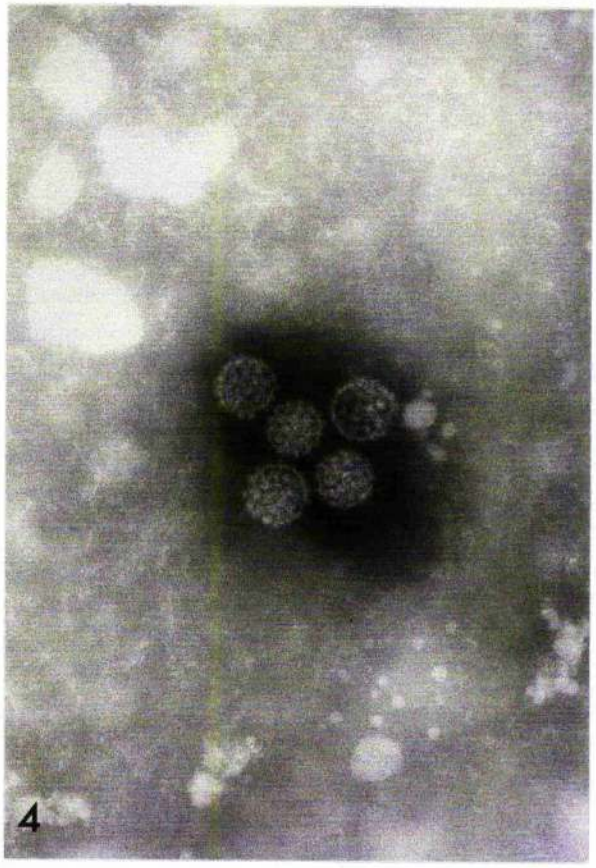
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Figures 5 and 6 are of preparations of spontaneously detached cells from control, uninoculated FRhK-4 cultures after incubation for 99 (Fig 5) and 155 (Fig 6) days; media contained 2% fetal bovine serum.

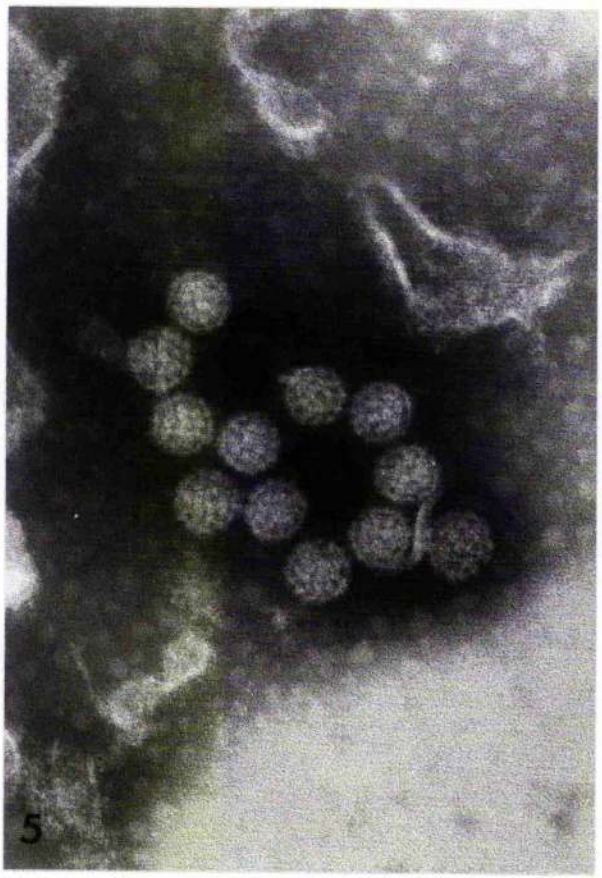
Fig 5. Three polyomavirions in this group are attached to a fragment of membrane and fine amorphous substance can be seen between some of the virus particles. x 200,000.

Fig 6. A "mini" polyomavirus particle is illustrated, together with three "normal" polyomavirions, attached to one of which is a small, featureless particle. x 200,000.

Figures 7 and 8 are of high speed pellets of culture fluids from the same bottle of control, uninoculated FRhK-4 cells (pass 81) harvested at different times.

Fig 7. FRhK-4 culture seven days after subdivision; the cells were on growth medium, which contained 10% fetal bovine serum. Two polyomavirus particles are surrounded by a dense coat of antibody and one possible polyomavirion is encased by membrane. x 200,000.

Fig 8. FRhK-4 culture 25 days after subdivision; the cells had been on maintenance medium, which contained 2% fetal bovine serum, for 18 days. Variable but smaller amounts of antibody than those in Figure 7 are attached to four polyomavirus particles. x 200,000.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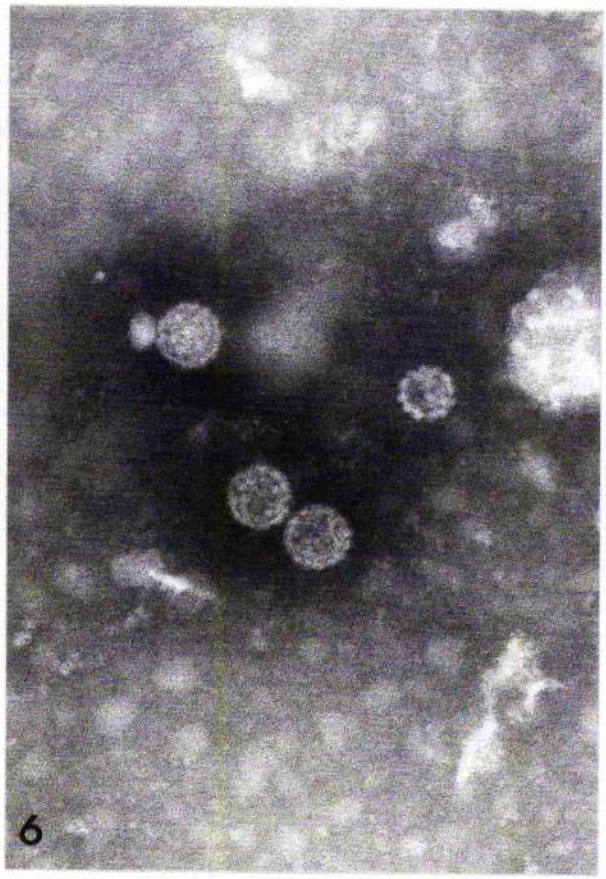
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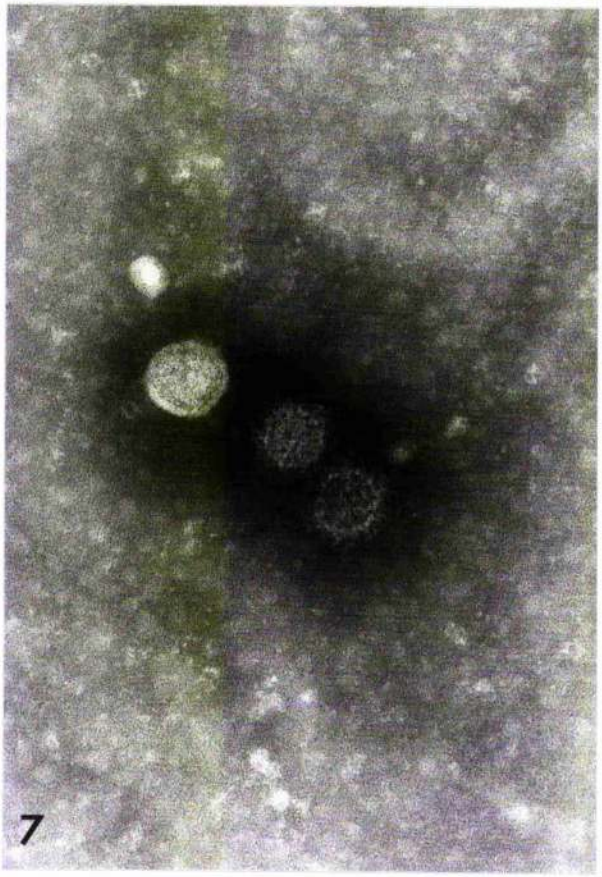
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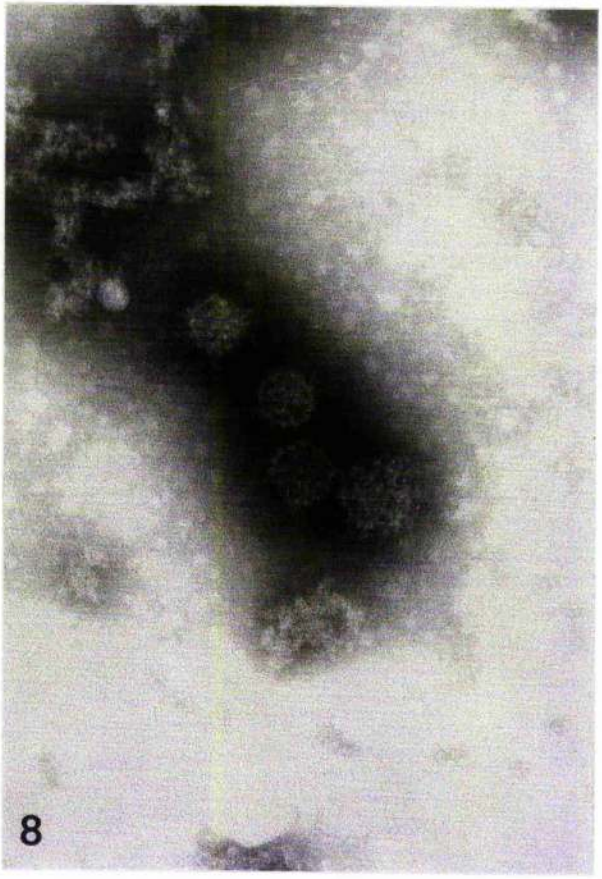
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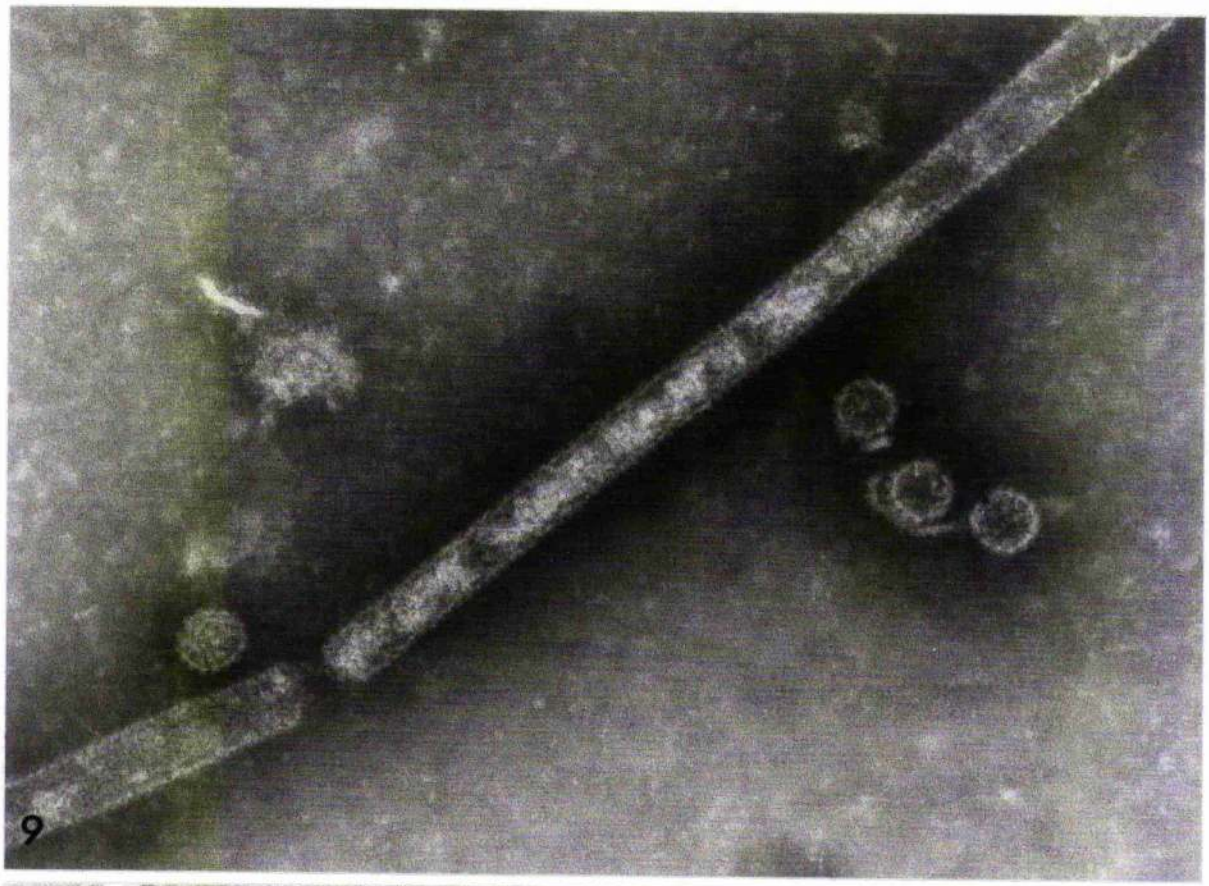
Figures 9 to 11 are of a preparation of spontaneously detached cells from uninoculated FRhK-6 culture (pass 7) after incubation for 21 days; medium contained 2% fetal bovine serum.

Fig 9. Polyomavirions, three of which are associated with membrane, lie near this portion of a long polyomavirus filament. x 200,000.

Fig 10. Several polyomavirions a few of which are damaged, a "mini" polyomavirus and four small, round, featureless particles can be seen. Some of the virus particles are associated with fine strands or amorphous substance. x 200,000.

Fig 11. Polyomavirions, part of a polyomavirus filament and two small, featureless particles are present. x 200,000.



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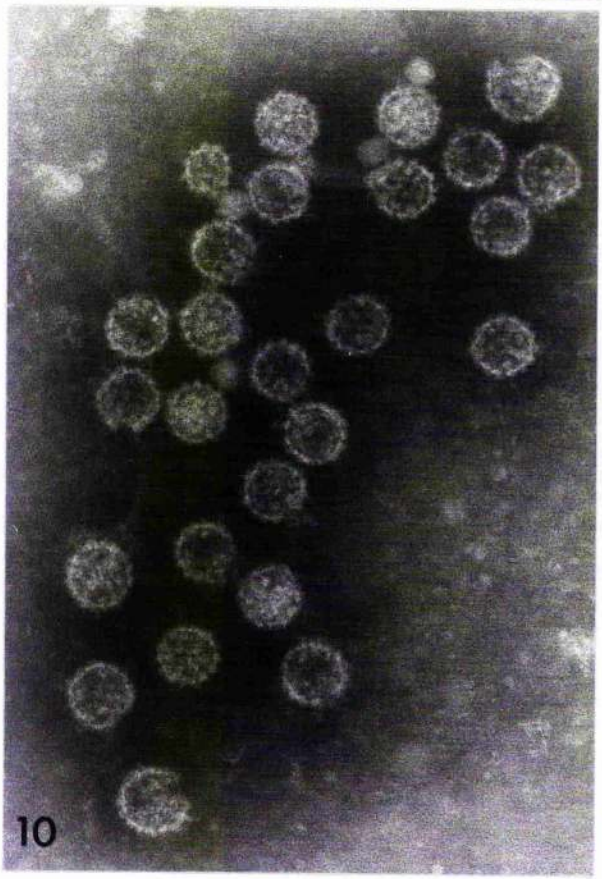

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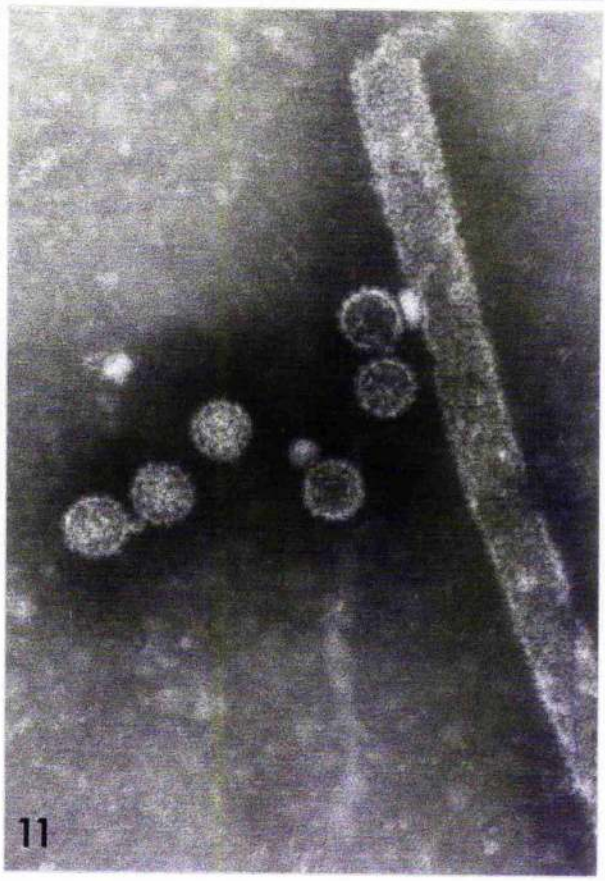

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Figures 12 and 13 are of high speed pellets of clarified culture fluids; medium contained 2% fetal (Fig 12) or newborn (Fig 13) bovine serum.

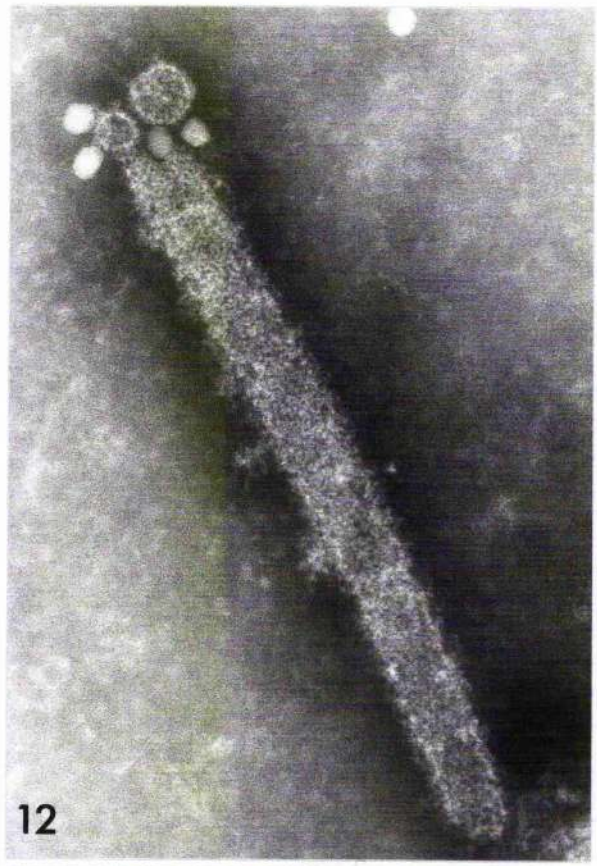
Fig 12. Uninoculated FRhK-6 culture (pass 7) after incubation for 21 days. Antibody partially obscures the morphology of a piece of polyomavirus filament and a "mini" particle, whereas one virus particle does not appear to have antibody attached. Four small, round particles are also present. x 200,000.

Fig 13. Uninoculated FRhK-4 culture (pass 25, USA). A clump of polyomavirions and small, round particles can be seen in this preparation. x 200,000.

Figures 14 and 15 are of high speed pellets of culture fluids; medium contained 2% fetal (Fig 14) or newborn (Fig 15) bovine serum.

Fig 14. Uninoculated FRhK-4 culture (pass 44, USA); after incubation for 21 days. Four polyomavirus particles are surrounded by a dense coating of antibody molecules. x 200,000.

Fig 15. DBS-FRhL-2 103 culture 28 days after inoculation with FRK4V. There is no evidence of antibody or amorphous substance associated with these two polyomavirions. x 200,000.



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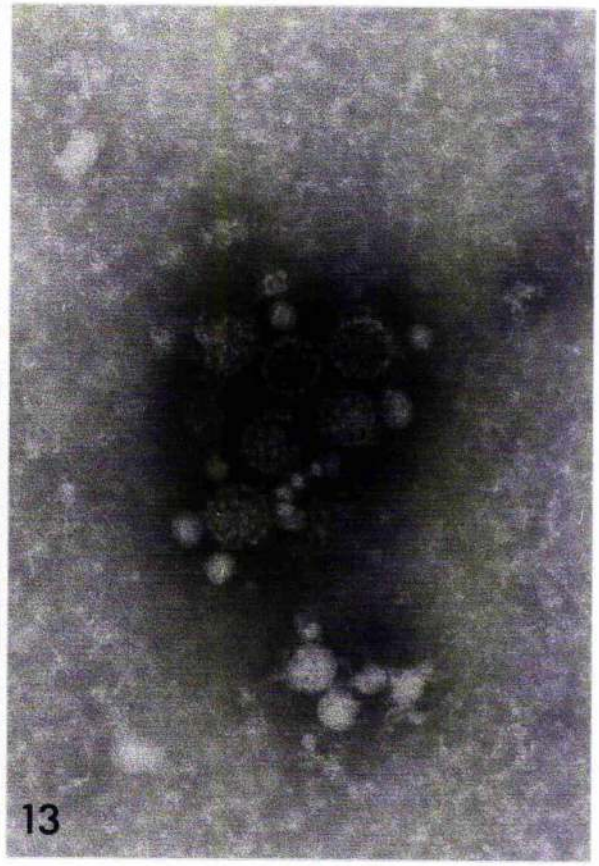

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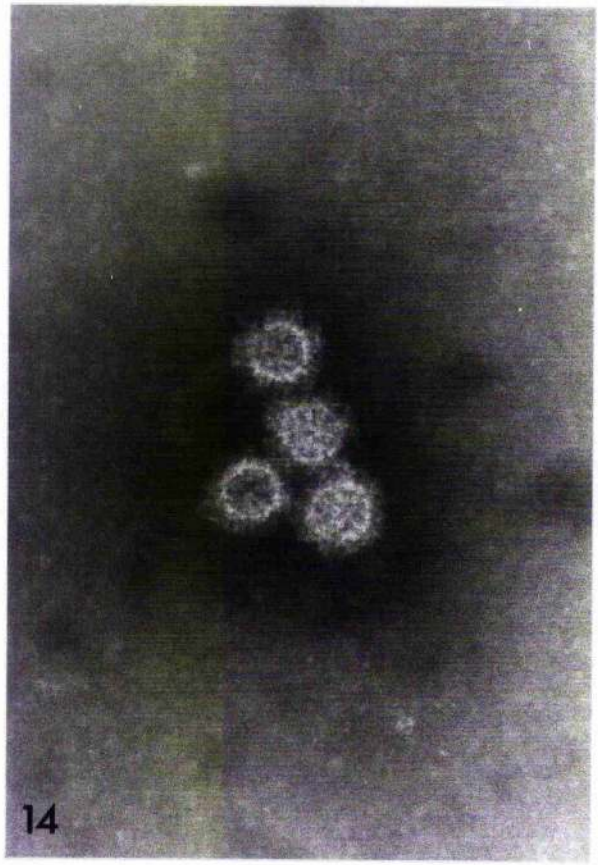

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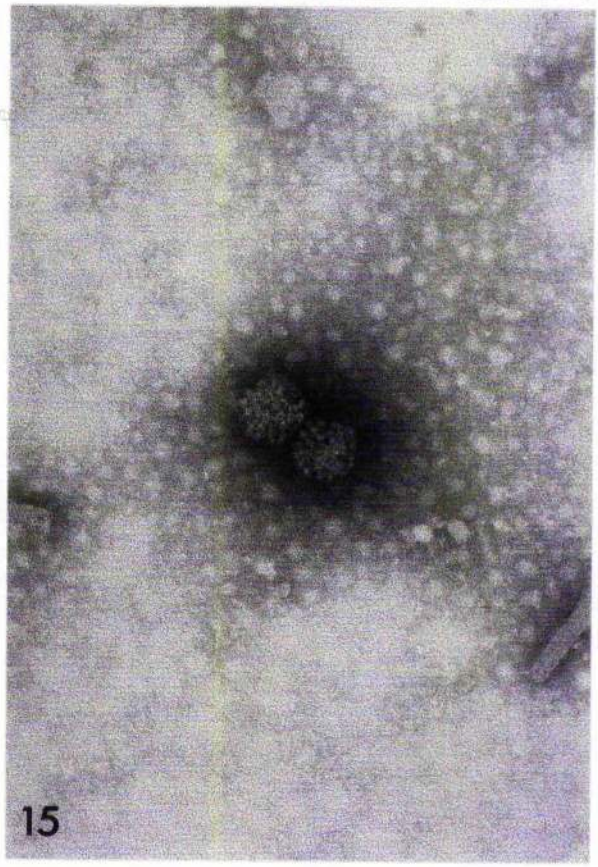

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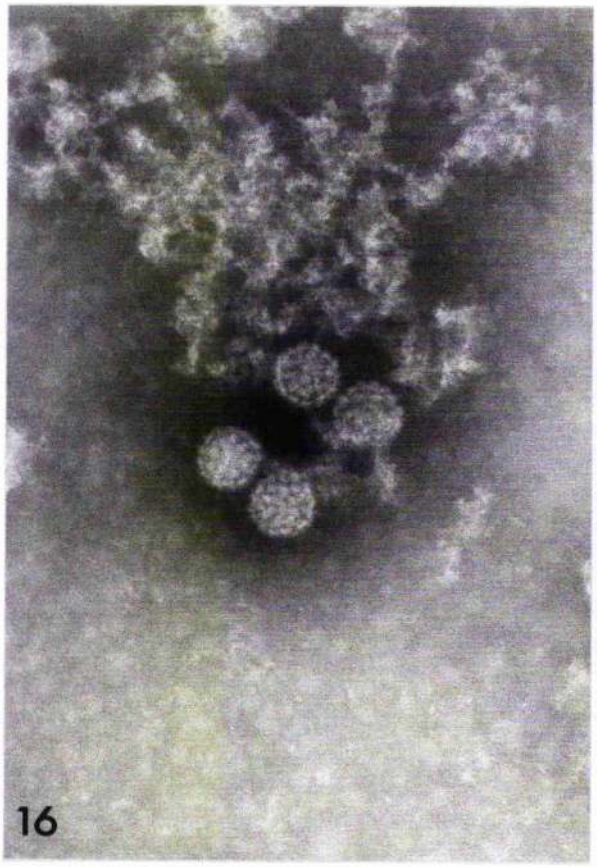
Figures 16 to 19 are of high speed pellets of culture fluids from HEK cells 16 (Fig 16) and 28 (Figs 17 to 19) days after inoculation with FRK4V; media contained 2% newborn bovine serum 17/8.

Fig 16. Amorphous substance is associated with polyomavirions. x 200,000.

Fig 17. Phosphotungstic acid has penetrated the membrane clearly revealing the structure of most of these membrane-bound polyomavirions. Where penetration of stain is less efficient, however, little more than the outline of the virus particles can be seen inside the membrane. x 200,000.

Fig 18. A clump of polyomavirions with dense antibody coating is illustrated. Some amorphous substance can also be seen. x 200,000.

Fig 19. Densely antibody-coated polyomavirus particles are associated with granular amorphous substance. x 200,000.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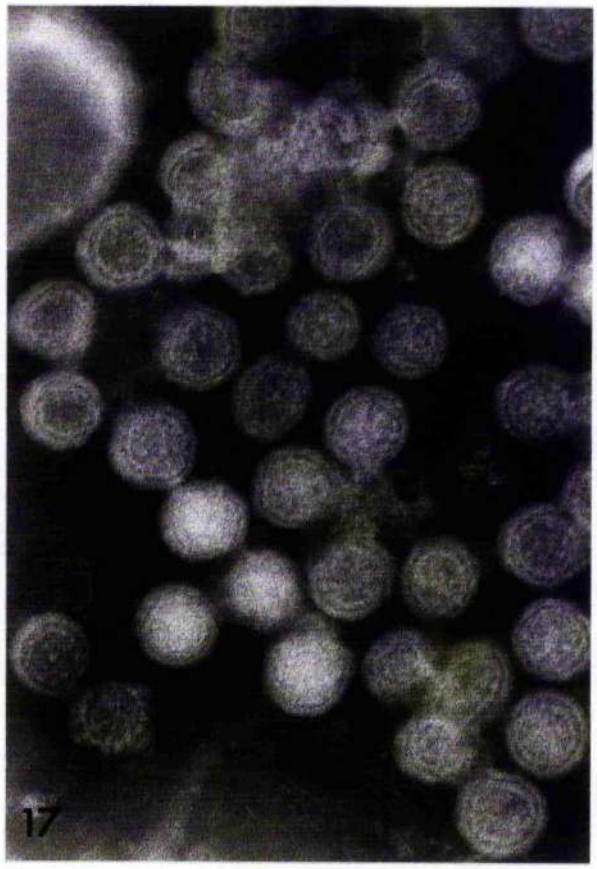
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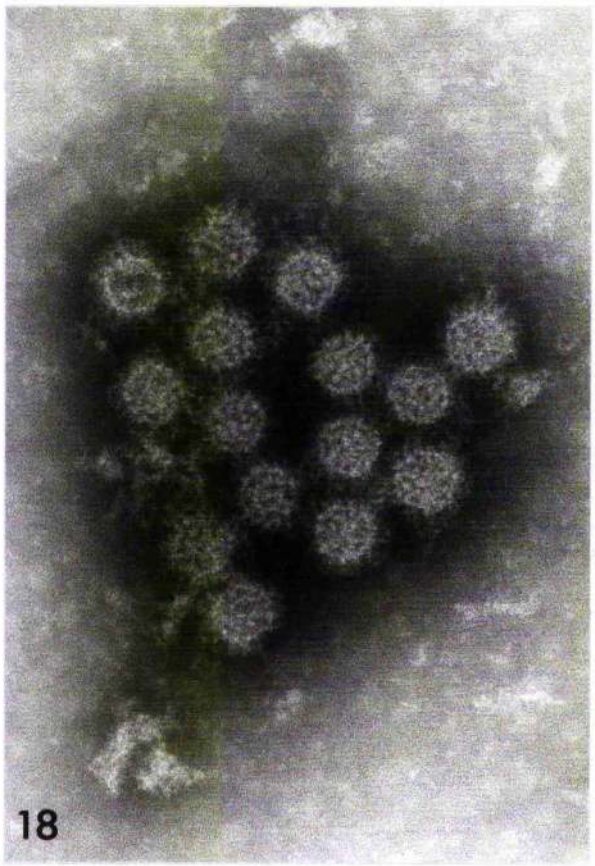
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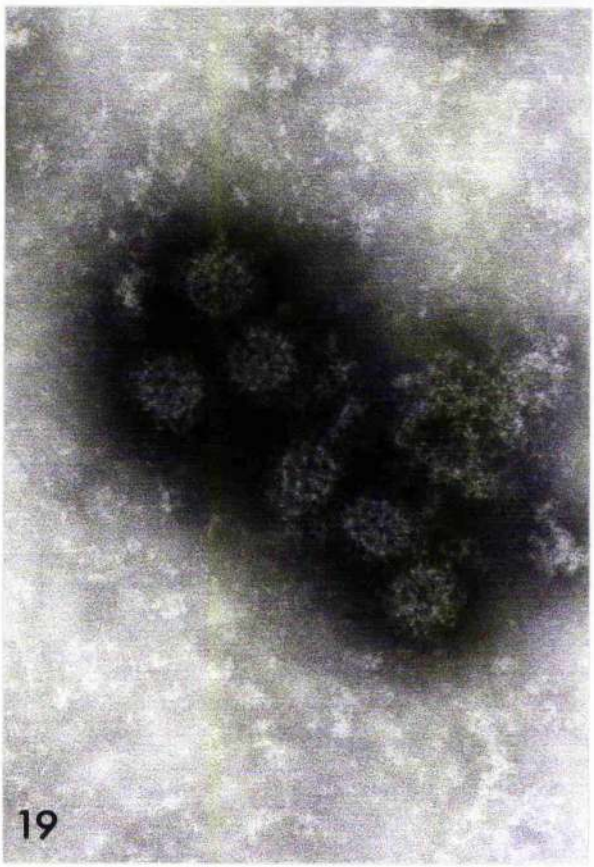
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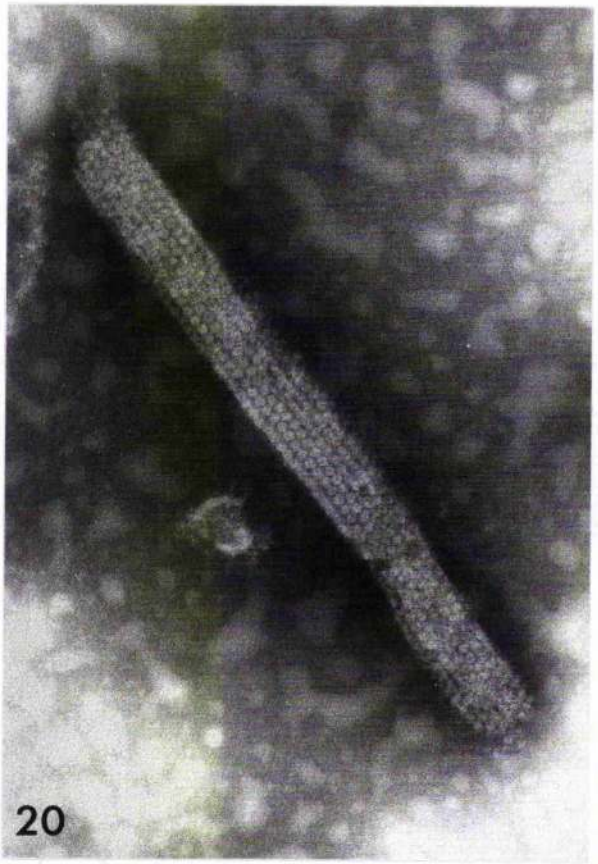
Figures 20 and 21 are of extracts from HEK monolayer cells 27 (Fig 20) and 62 (Fig 21) days after inoculation with FRK4V. Although the media contained 2% newborn bovine serum 17/8, evidence of antibody could not be found in such preparations.

Fig 20. The capsomers are particularly clear in this piece of polyomavirus filament. x 200,000.

Fig 21. Three virus particles are attached to a fragment of membrane. x 200,000.

Fig 22. High speed pellet of culture fluid from CK8 cells ten days after inoculation with HEK-adapted FRK4V; medium contained 2% newborn bovine serum. Large numbers of polyomavirions occur singly and in clumps, many of which include small round particles. Three long polyomavirus filaments and amorphous substance are also present. x 30,000.



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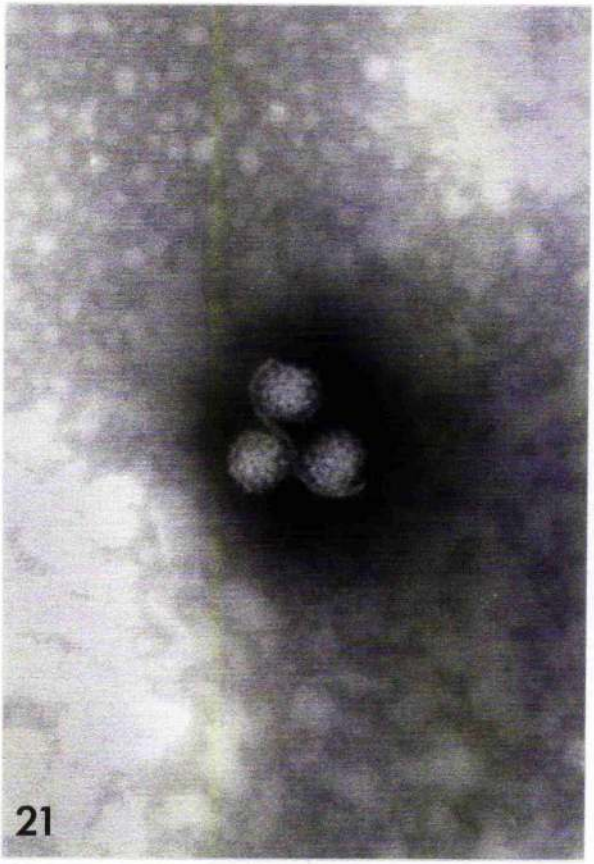

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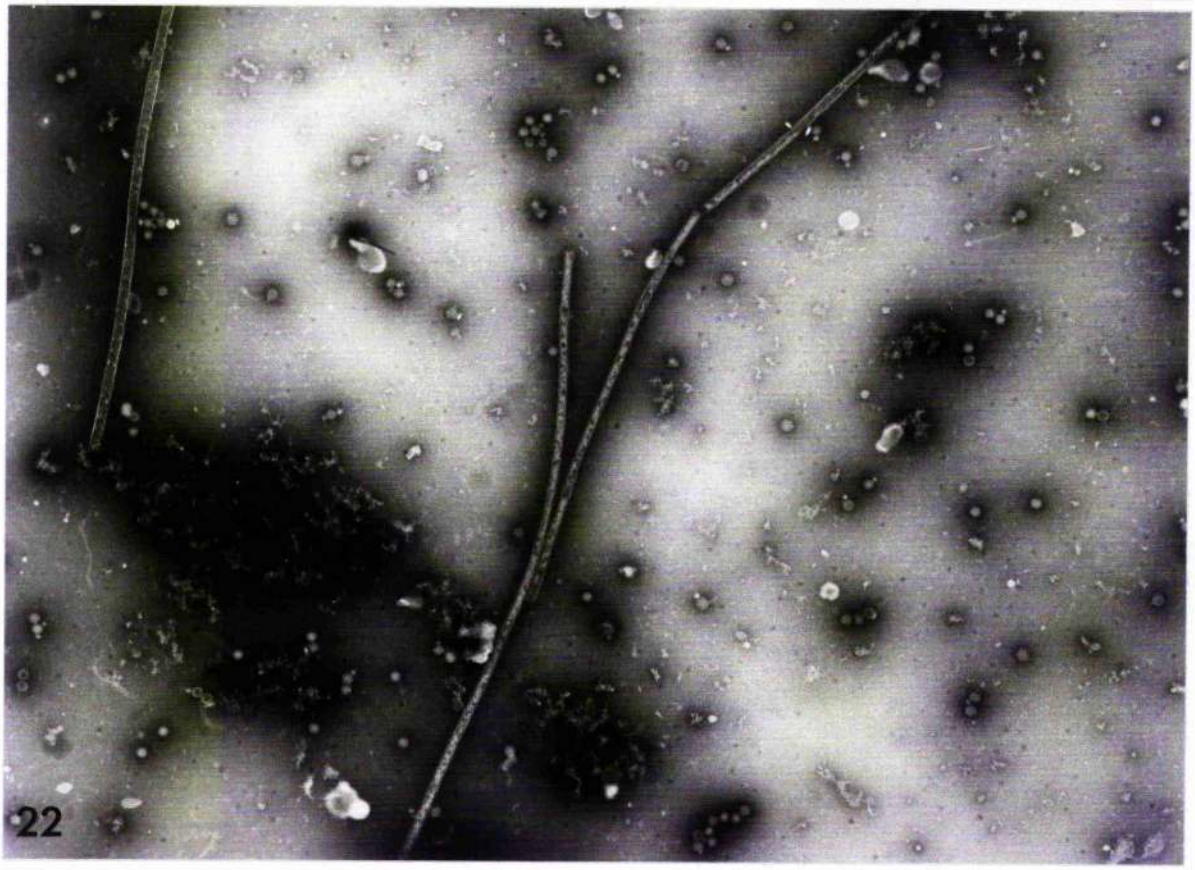


22

Figures 23 and 24 are of preparations of spontaneously detached cells from CK17 cultures; media contained 2% fetal bovine serum.

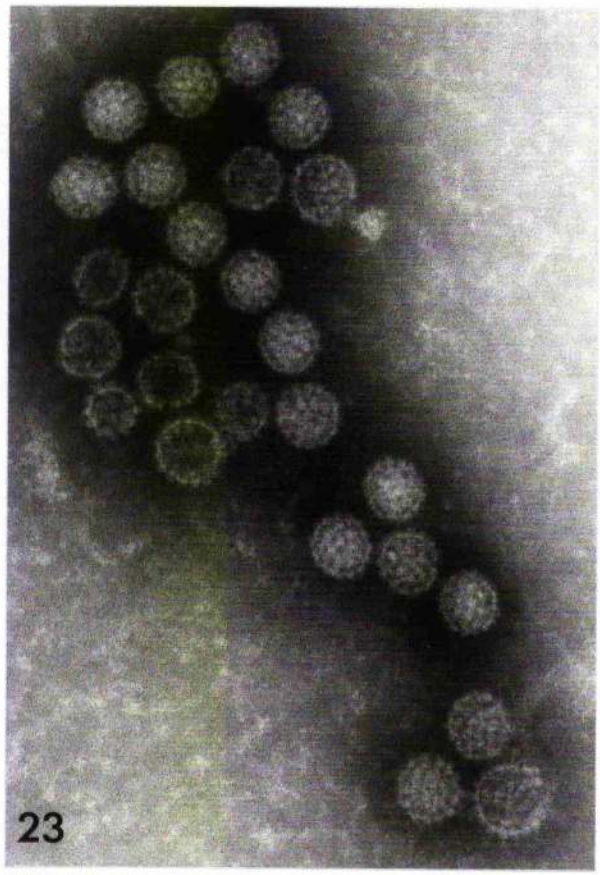
Fig 23. CK17 cells 59 days after inoculation with FRK6V. Several polyomavirions, including "mini" particles, are present in groups. Although antibody cannot be identified, fine strands seem to be holding some of the virions together. x 200,000.

Fig 24. Control, uninoculated CK17 cells after incubation for 59 days. Two "clean" polyomavirions can be seen. x 200,000.

Figures 25 and 26 are of preparations of FRhK-4 cultures 12 (Fig 25) and 22 (Fig 26) days after the cells had been maintained on Iscove's serum-free medium.

Fig 25. Spontaneously detached cells. There is some indication of fine strands of amorphous substance between the polyomavirions in this clump. x 200,000.

Fig 26. High speed pellet of clarified culture fluid. Three single, "clean" polyomavirus particles and coarse or granular amorphous substance are present. x 200,000.



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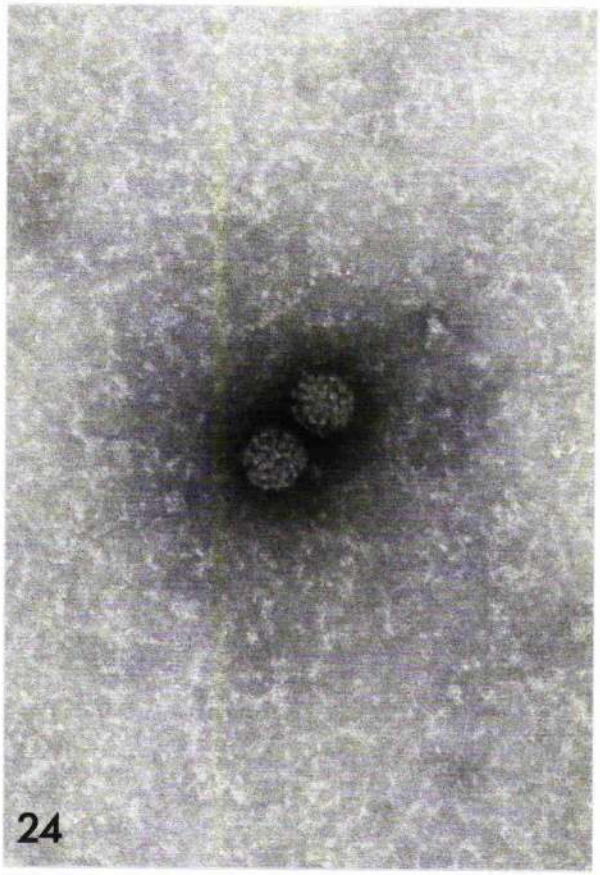

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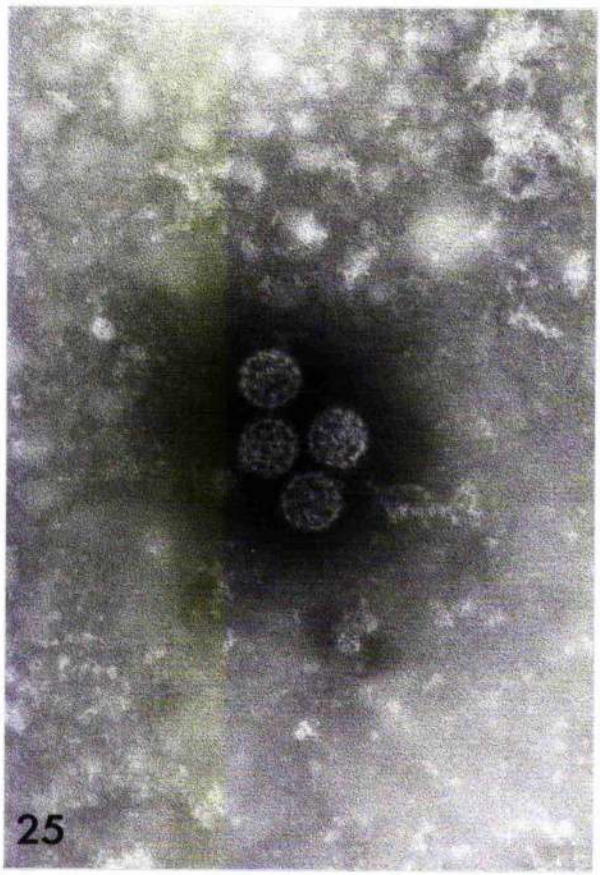

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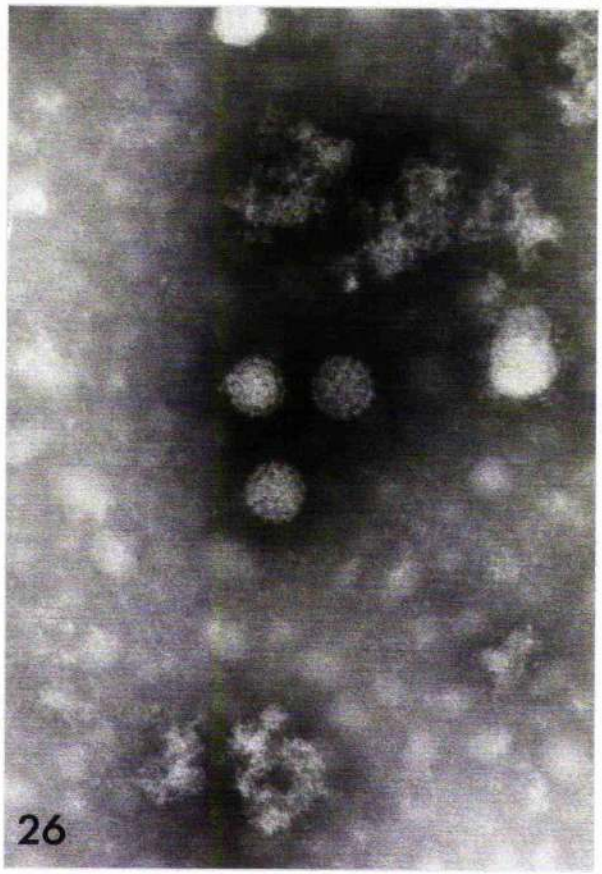


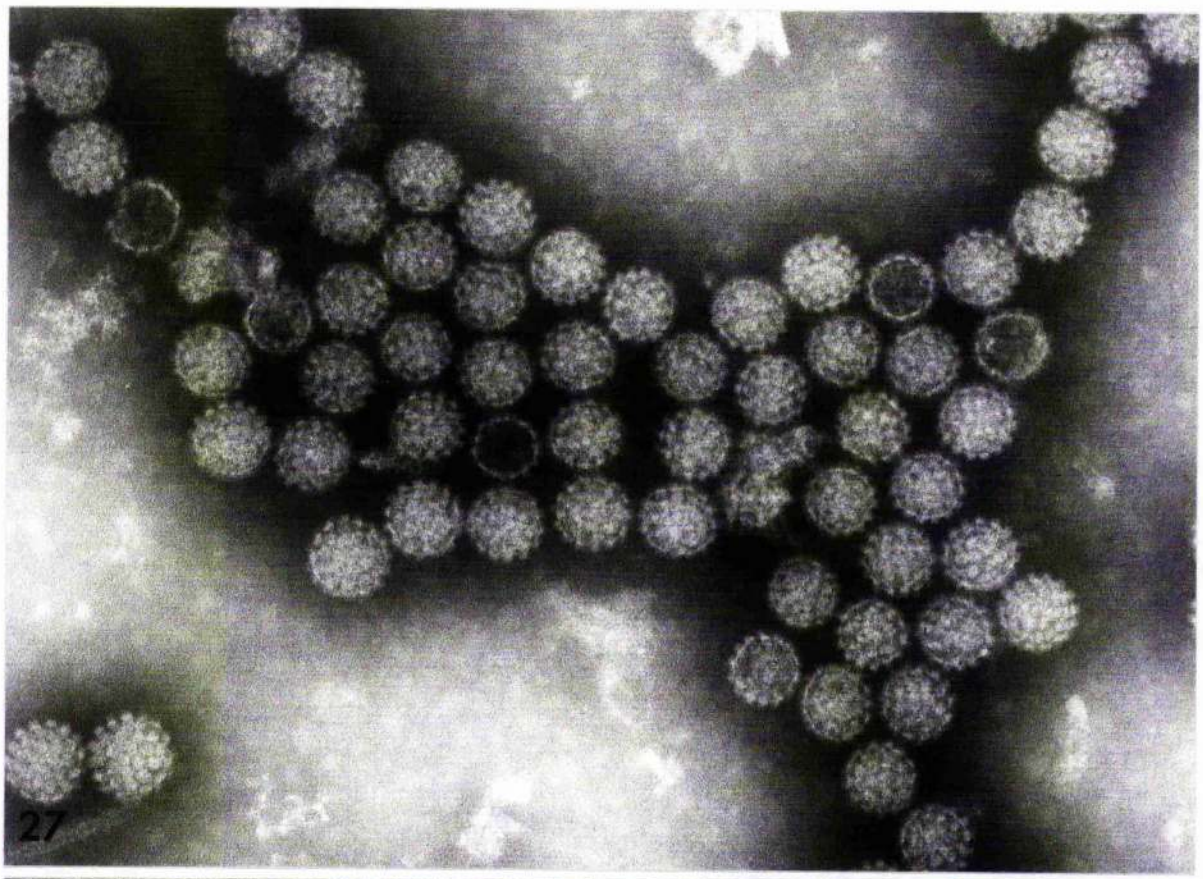
26

Figures 27 to 29 are of a preparation of human papillomavirus partially purified from plantar wart.

Fig 27. A large number of papillomavirus particles and some amorphous substance are illustrated. x 200,000.

Fig 28. Amorphous substance associated with papillomavirions is more evident here than in Figure 27. x 200,000.

Fig 29. In addition to spherical particles, there are three narrow papillomavirus filaments; amorphous substance is also present. x 200,000.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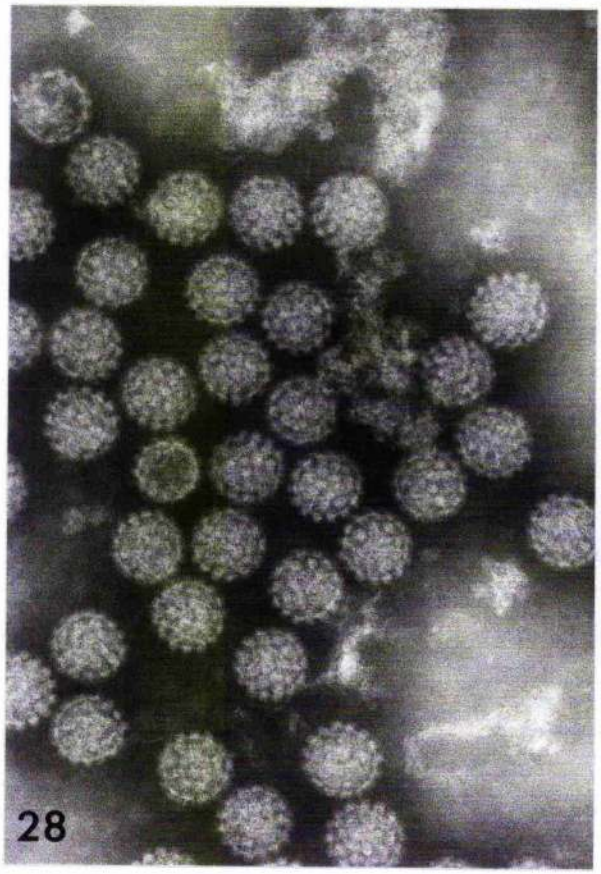
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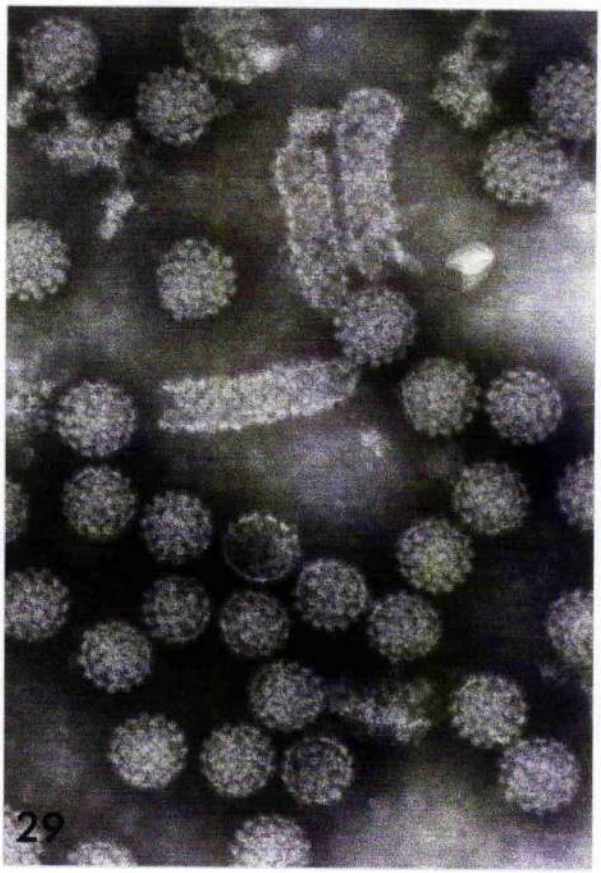
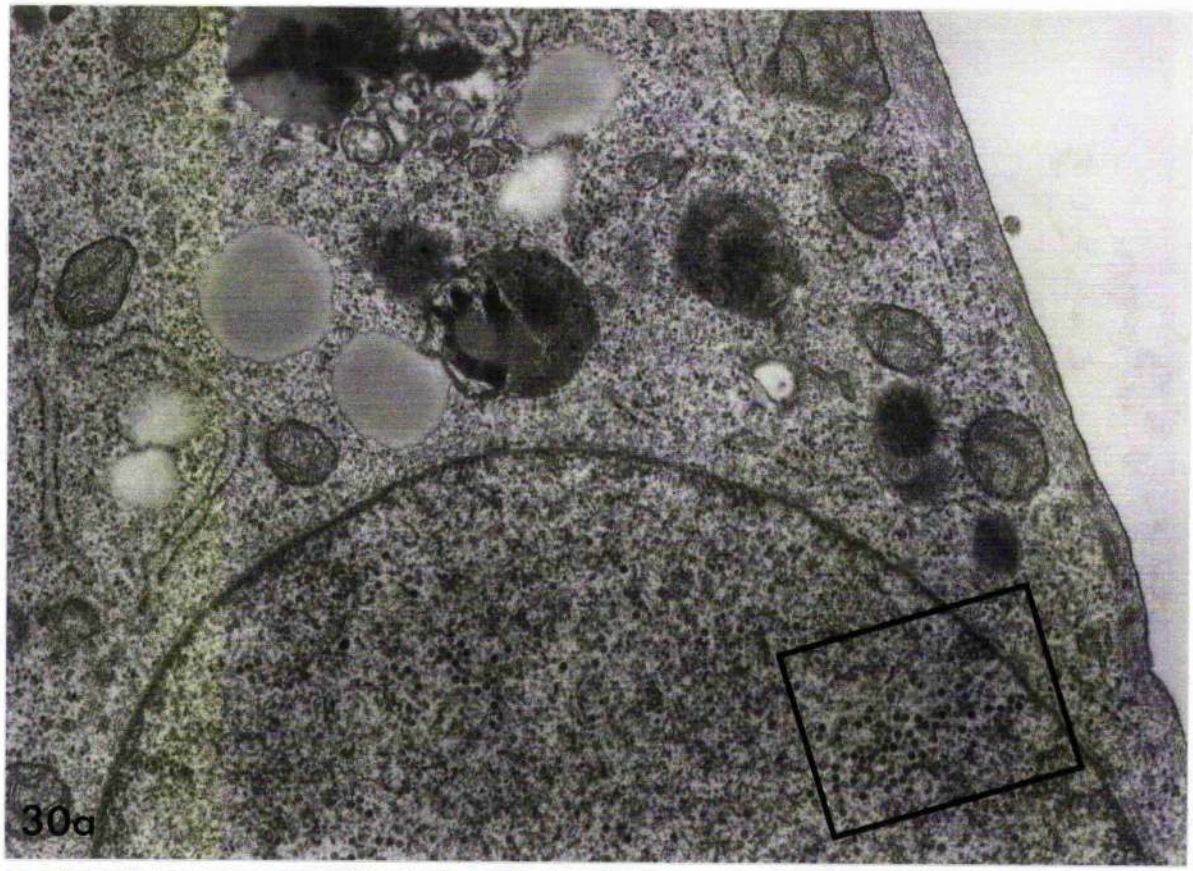

29

Figures 30 to 44 are electron micrographs of thin sections of FRhK-4 cultures. Monolayer cells in situ, or spontaneously detached cells in suspension, were sequentially fixed with glutaraldehyde followed by osmium tetroxide. The samples were dehydrated and embedded in epoxy resin. Thin sections were contrast stained with uranyl acetate then lead citrate.

Figure 30 is of FRhK-4 monolayer culture (pass 81), fixed in situ, 55 days after inoculation with HAV.

Fig 30a. Part of a morphologically intact cell is shown; polyomavirus particles are randomly organized in loose groups within the nucleus. x 30,000.

Fig 30b. A higher magnification of the polyomavirions in the area of nucleus outlined. x 150,000.


30a

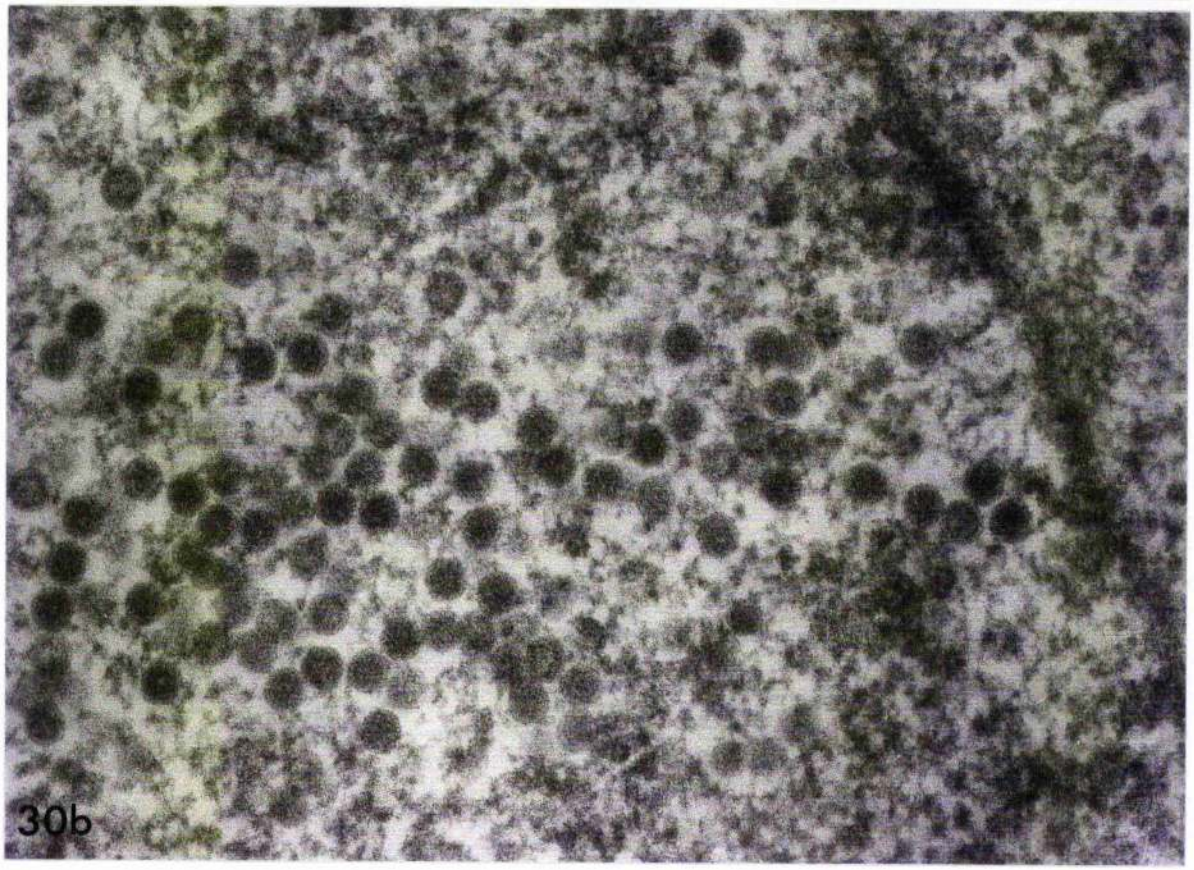
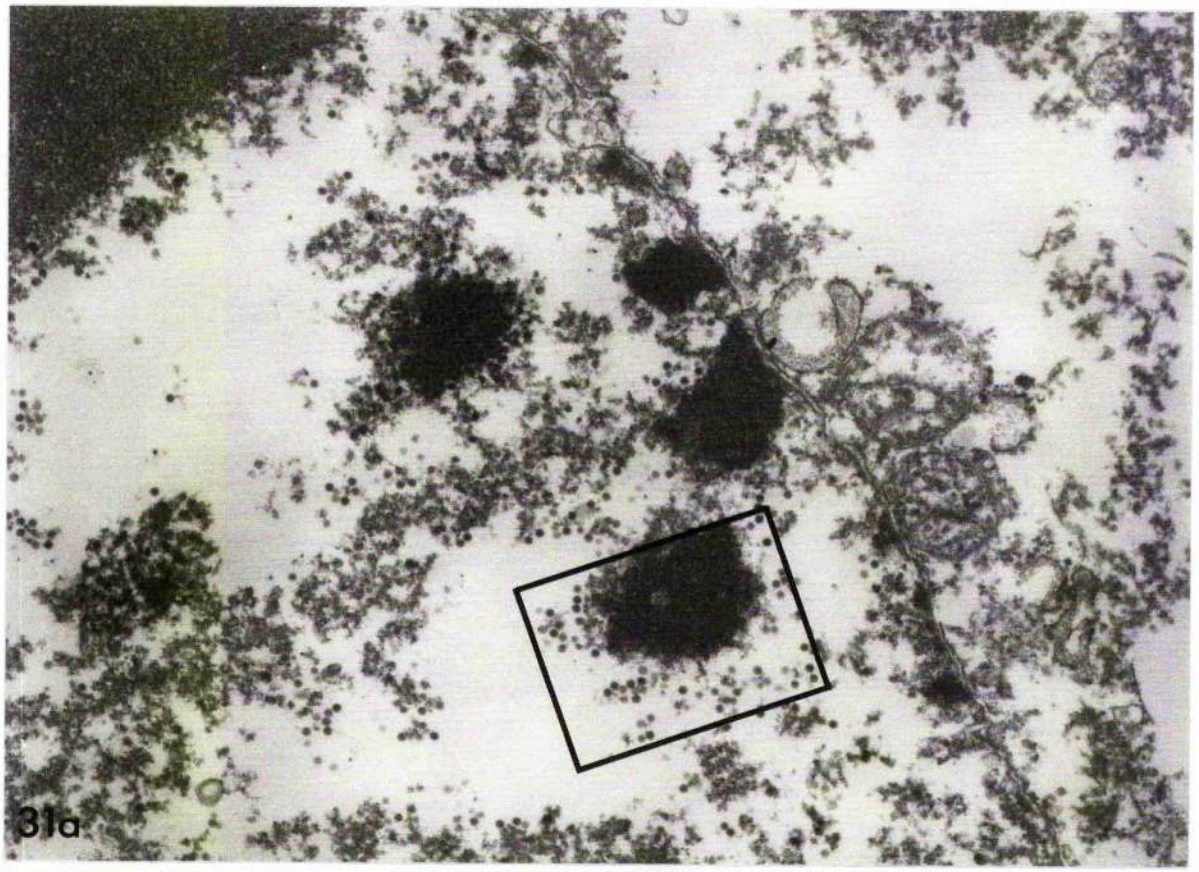

30b

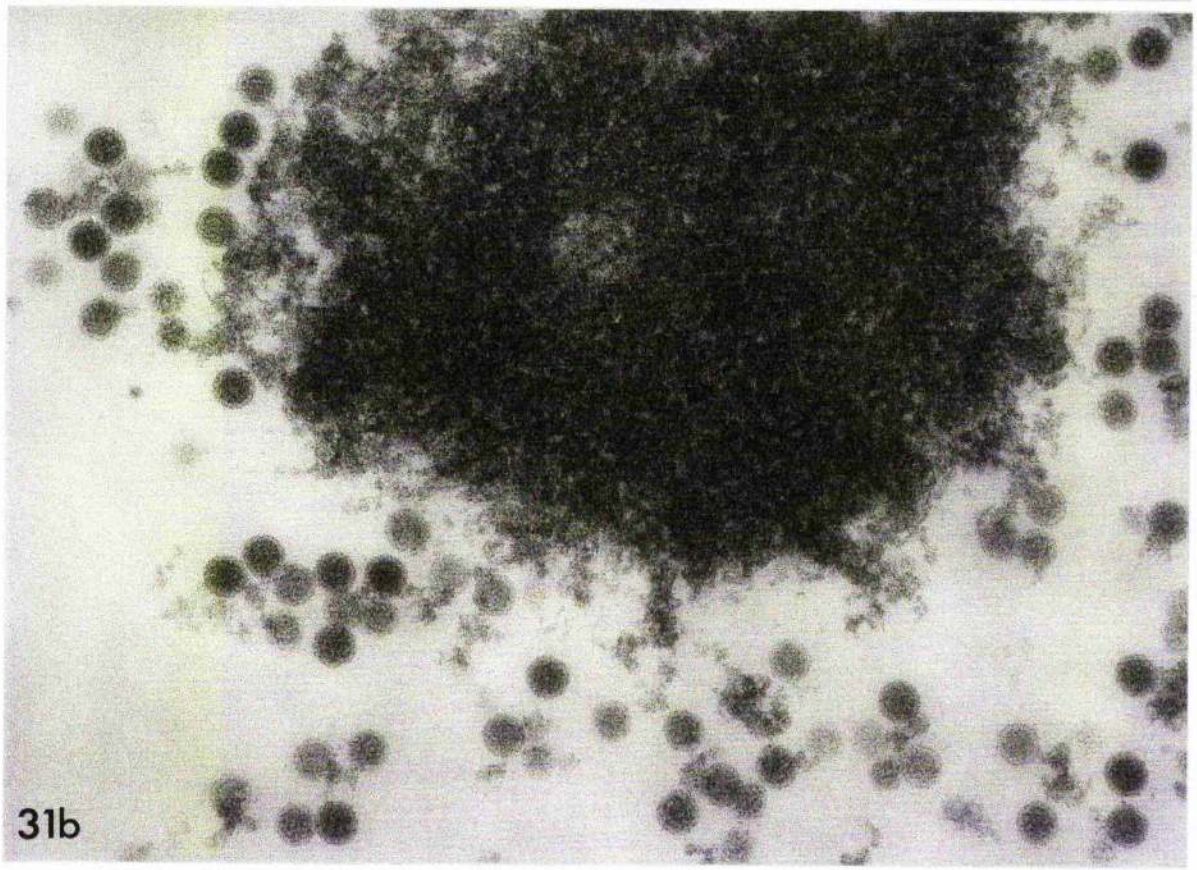
Figure 31 is of FRhK-4 monolayer culture (pass 81), fixed in situ, 55 days after inoculation with HAV.

Fig 31a. Intranuclear polyomavirions are more easily identified in this degenerate cell than in Figure 30a. x 30,000.

Fig 31b. A higher magnification of the area of nucleus outlined: many of the polyomavirus particles are associated with electron-opaque substance. x 150,000.



31a

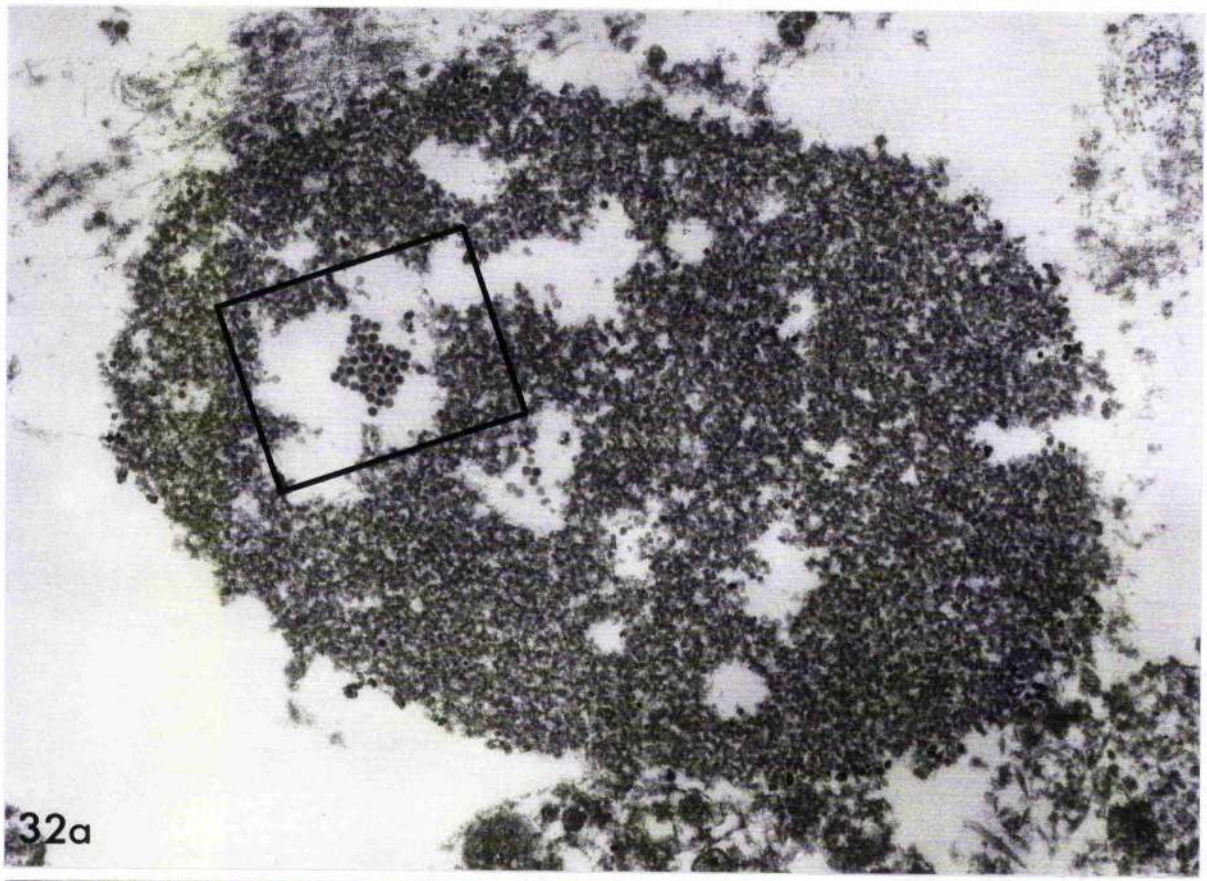


31b

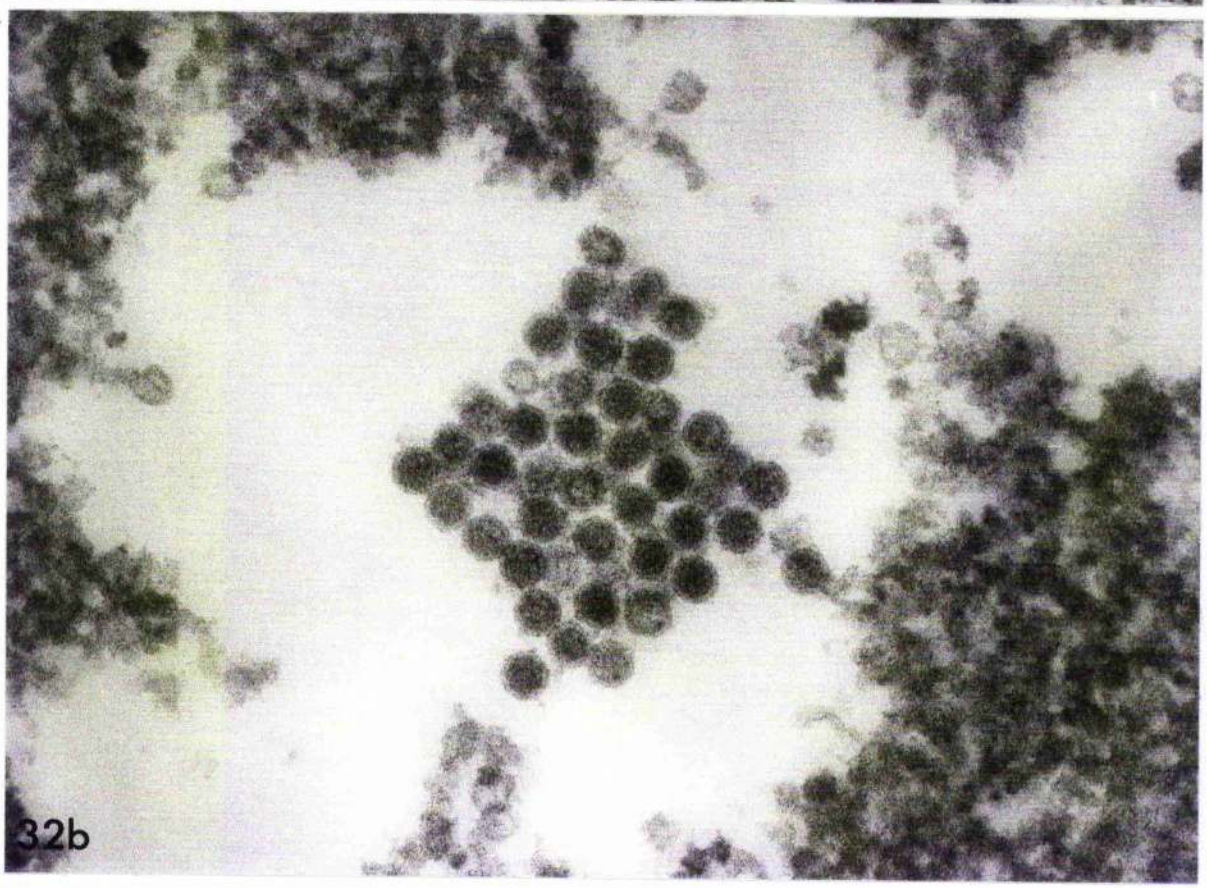
Figure 32 is of control, uninoculated FRhK-4 monolayer culture (pass 81), fixed in situ, after incubation for 55 days.

Fig 32a. A paracrystalline array of polyomavirions is surrounded by chromatin in this degenerate nucleus. x 30,000.

Fig 32b. A higher magnification of the area outlined illustrates the presence of electron-opaque substance between many of the polyomavirus particles. x 150,000.



32a



32b

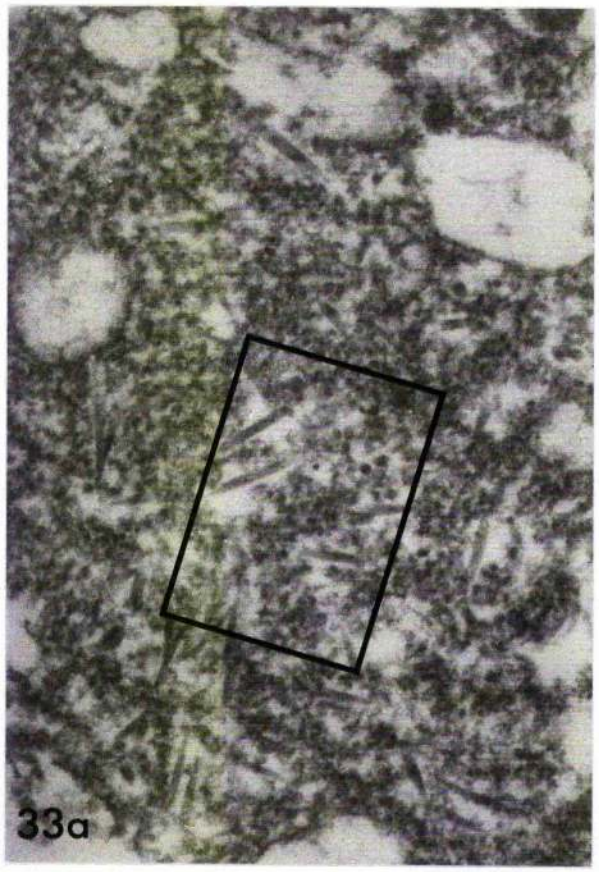
Figures 33 and 34 are of control, uninoculated FRhK-4 monolayer culture (pass 81), fixed in situ, after incubation for 55 days.

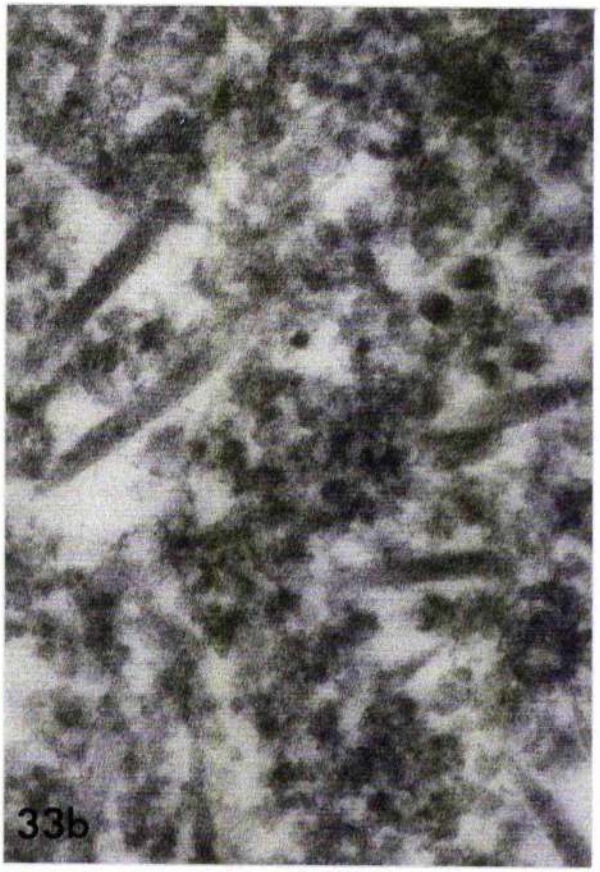
Fig 33a. Intranuclear polyomavirus filaments are illustrated. x 50,000.

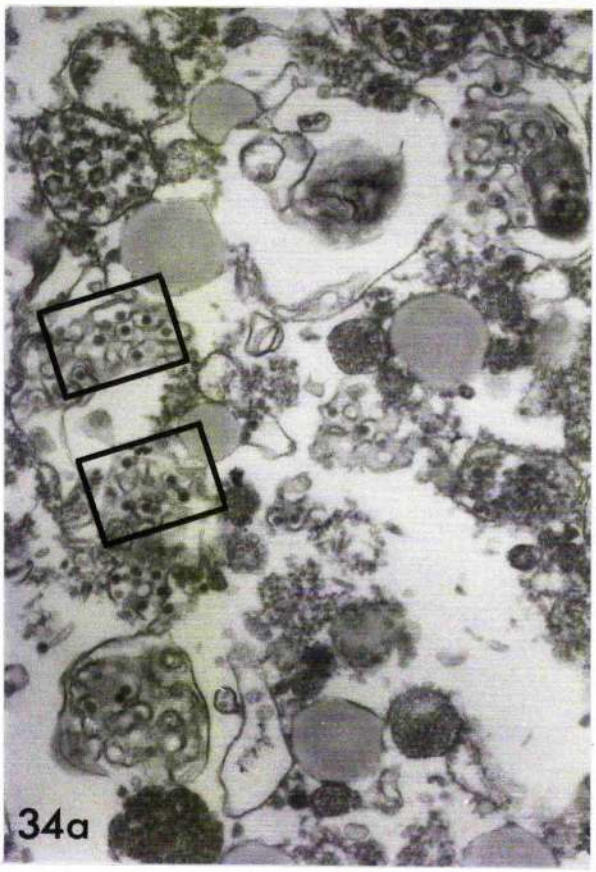
Fig 33b. A higher magnification of the area outlined in Figure 33a. x 150,000.

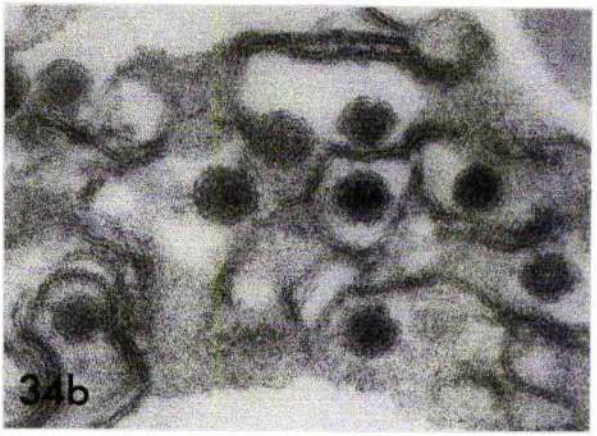
Fig 34a. Numerous polyomavirions are associated with cytoplasmic membranes. x 30,000.

Figs 34b & 34c. Higher magnifications of the areas outlined in Figure 34a demonstrate that some virus particles are loosely surrounded by membrane whereas others are closely enveloped. x 150,000.


33a


33b


34a


34b

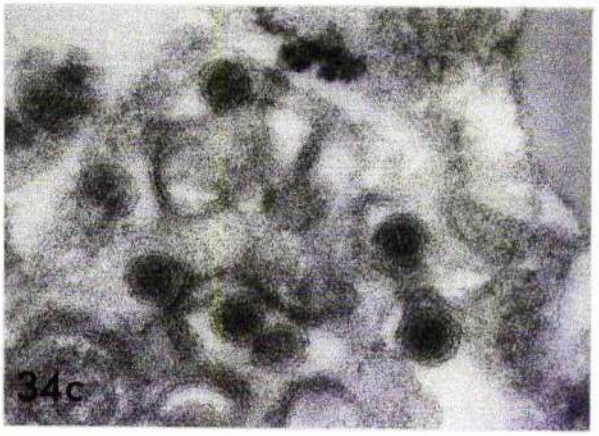
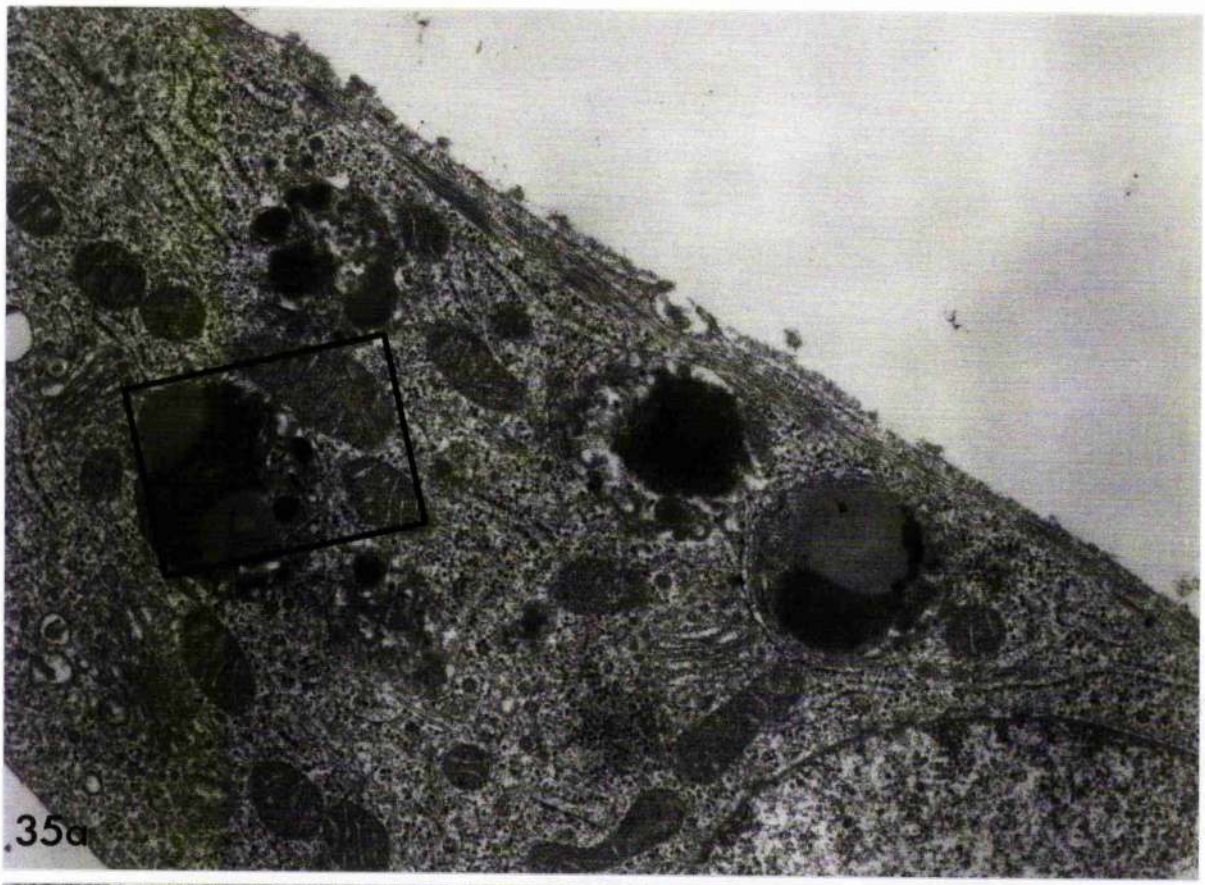

34c

Figure 35 is of FRhK-4 monolayer culture (pass 81), fixed in situ, 55 days after inoculation with HAV.

Fig 35a. Part of a morphologically intact cell is shown. In addition to normal cytoplasmic organelles, picornavirus-like particles associated with autophagic vacuoles can be observed. x 30,000.

Fig 35b. A higher magnification of part of the autophagic vacuole with picornavirus-like particles in the area outlined. x 150,000.


35a

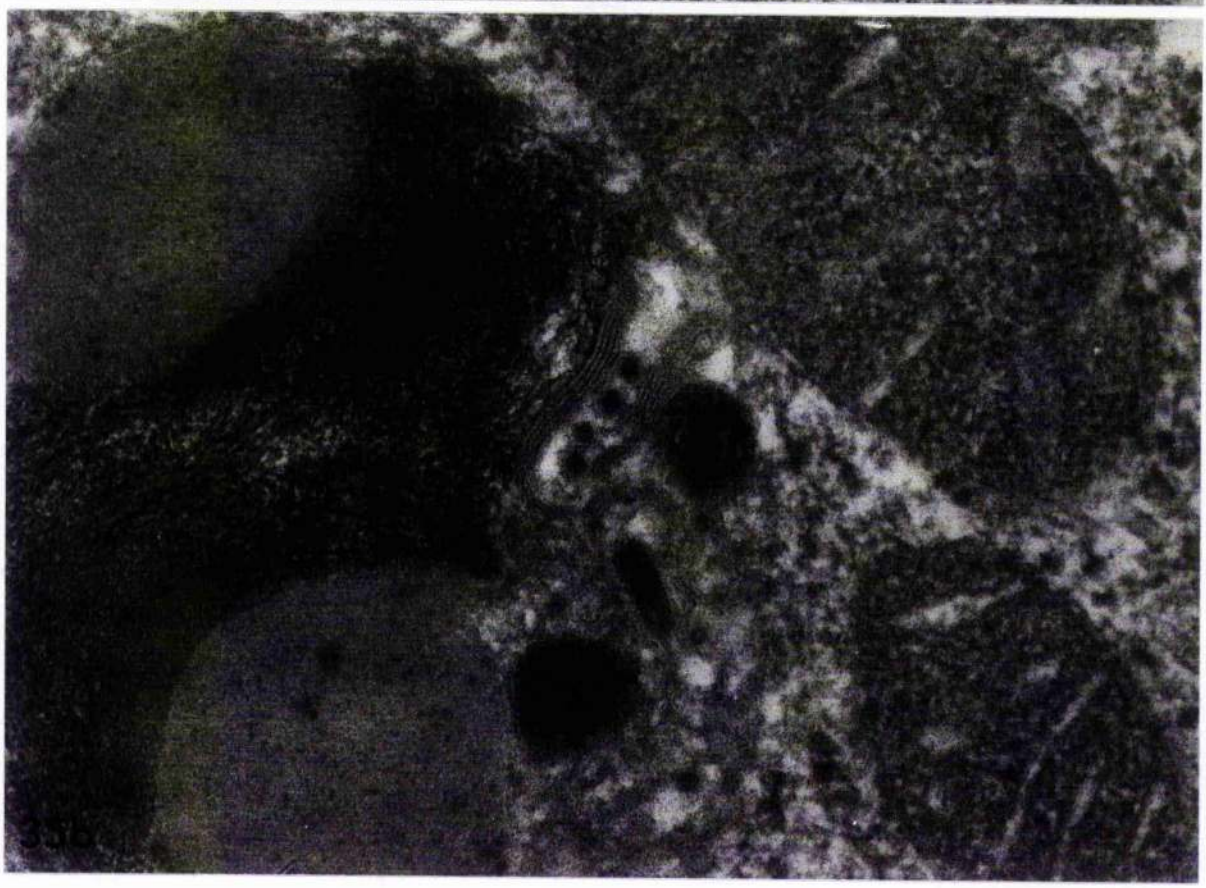
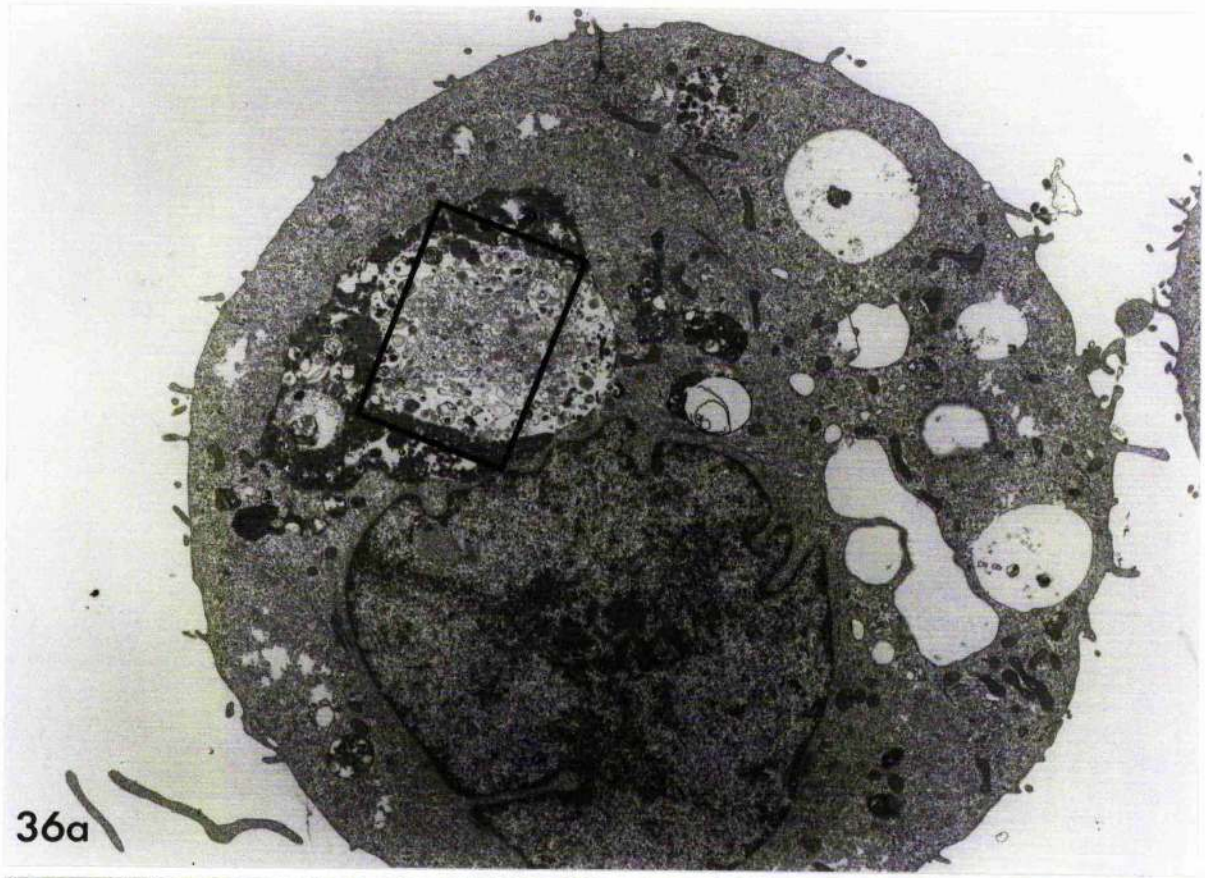


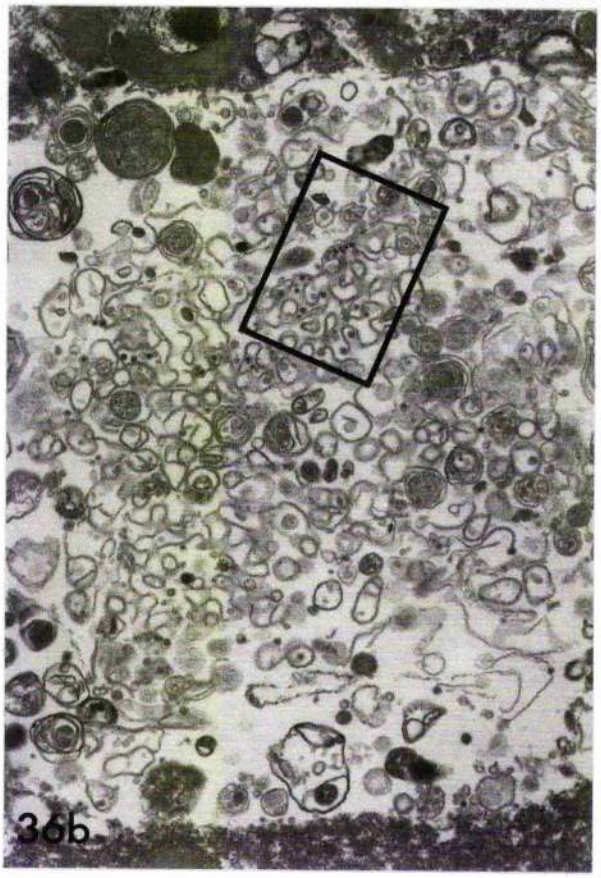
Figure 36 is of spontaneously detached cells from FRhK-4 monolayer culture (pass 81), fixed in suspension, 107 days after inoculation with HAV.

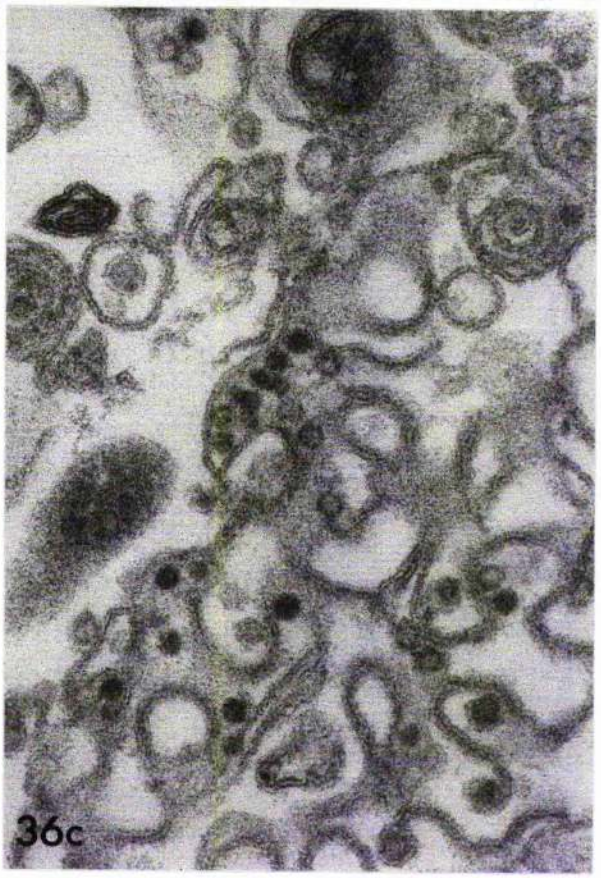
Fig 36a. The rounded profile of the cell is characteristic of FRhK-4 cells fixed in suspension compared with the appearance of favourably sectioned monolayer cells fixed in situ (Figs 30a & 35a). Autophagic and relatively empty vacuoles of various sizes are present. x 7,500.

Fig 36b. A higher magnification of the area outlined in Figure 36a. Numerous electron-opaque picornavirus-like particles are clearly visible amongst membranous whorls within this large autophagic vacuole. x 30,000.

Fig 36c. A higher magnification of the area outlined in Figure 36b. It is evident that the centres of the picornavirus-like particles are either electron-opaque or electron-translucent, which presumably correspond to the "full" and "empty" virus particles observed in negatively stained preparations. x 150,000.


36a


36b

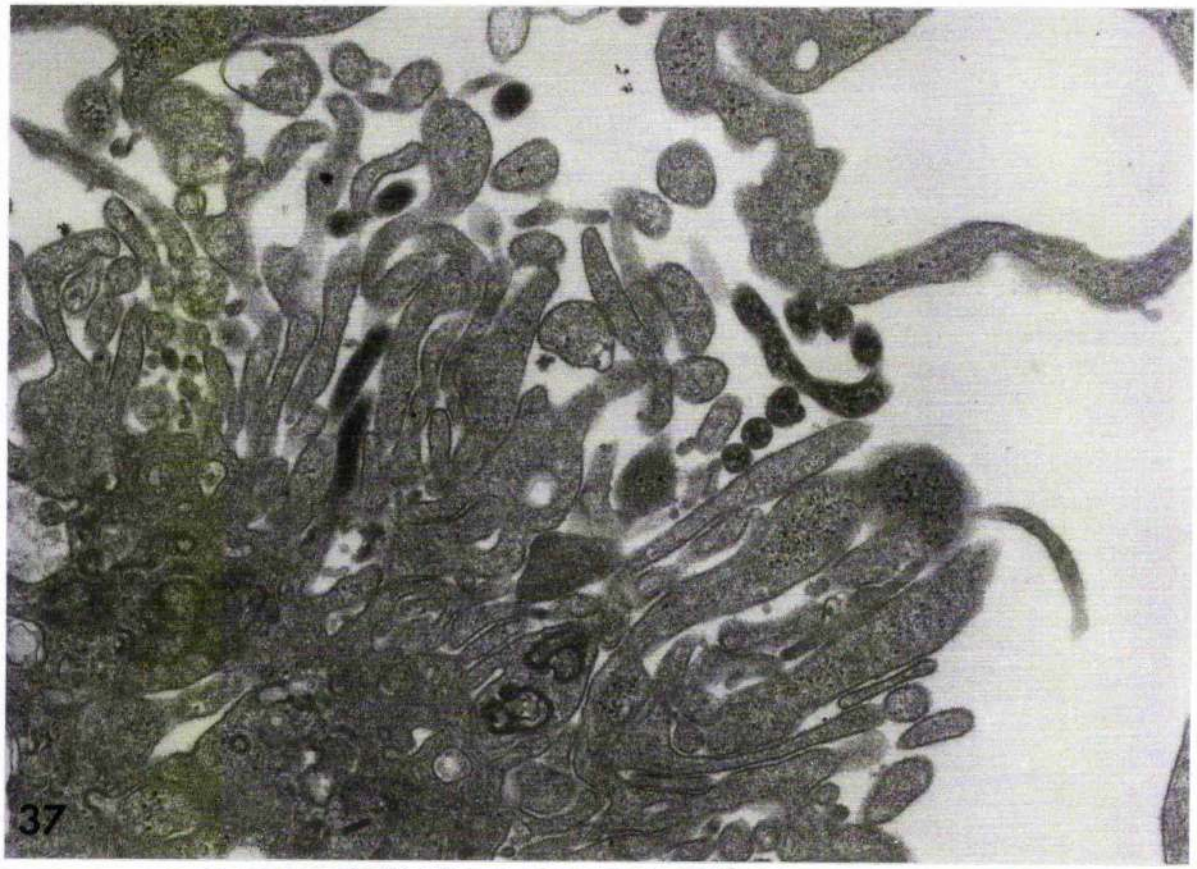

36c

Figures 37 and 38 are of FRhK-4 culture (pass 81) 107 days after inoculation with HAV. Monolayer cells fixed in situ (Fig 37) and spontaneously detached cells fixed in suspension (Fig 38).

Fig 37. Mycoplasmas interweave between the surface projections of this FRhK-4 cell. x 30,000.

Fig 38a. Numerous picornavirus-like particles, some of which are associated with membrane, are visible amongst the cytoplasmic organelles of this degenerate cell. x 30,000.

Fig 38b. A higher magnification of the area outlined further illustrates the electron-opaque or electron-translucent nature of the centres of the picornavirus-like particles. x 150,000.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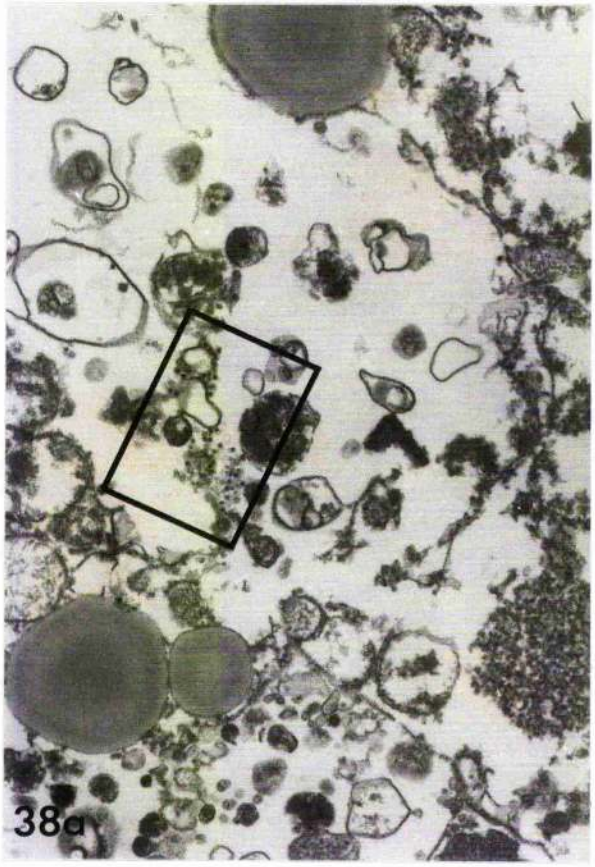
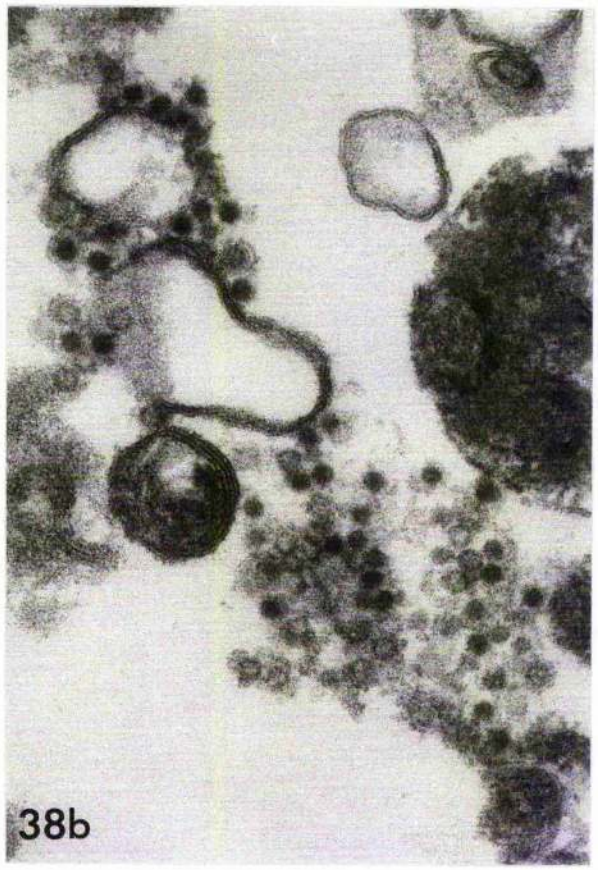

38a


38b

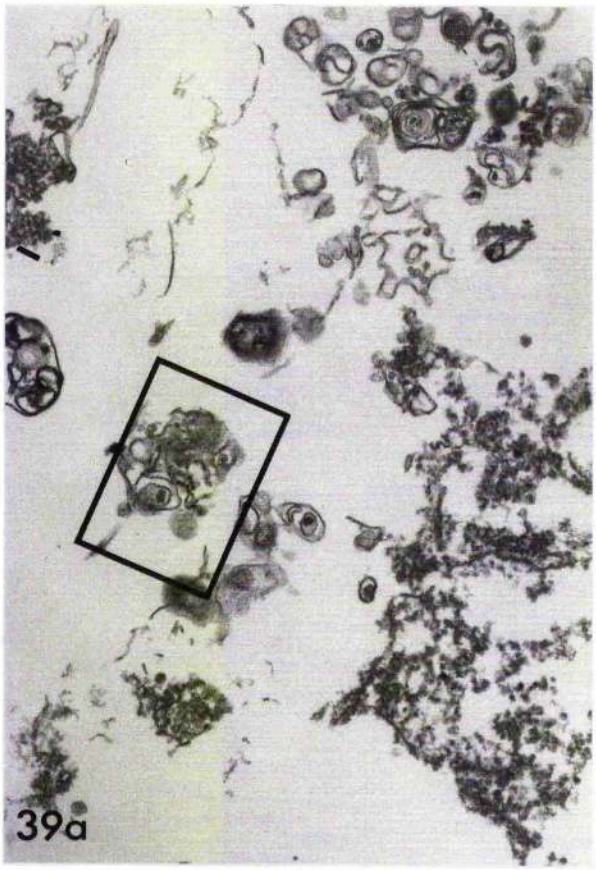
Figures 39 and 40 are of spontaneously detached cells from FRhK-4 culture (pass 81), fixed in suspension, 128 days after inoculation with HAV.

Fig 39a. Cytoplasmic picornavirus-like particles, in a group, are associated with membrane in a degenerate cell. x 30,000.

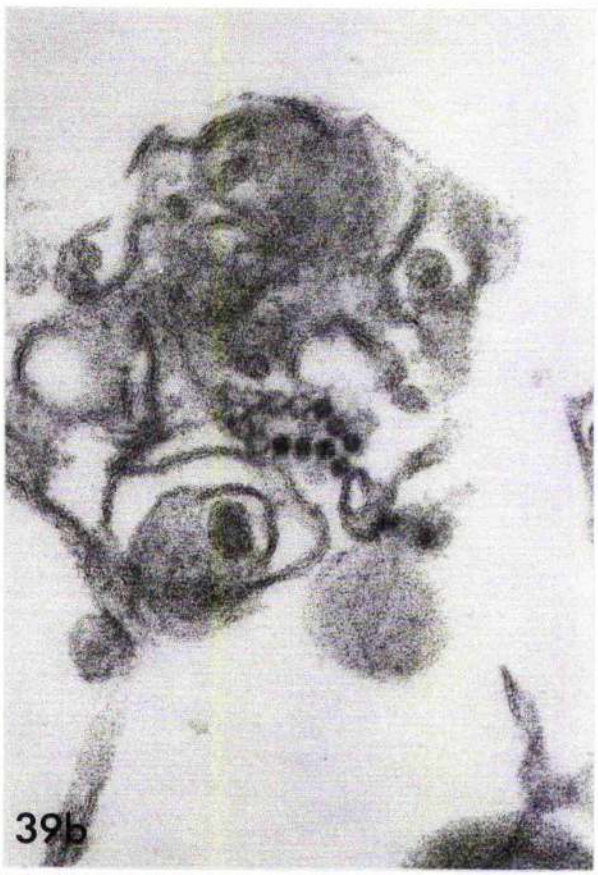
Fig 39b. A higher magnification of the electron-opaque and electron-translucent picronavirus-like particles in the area outlined in Figure 39a. x 150,000.

Fig 40a. Scattered picornavirus-like particles can be identified within this collection of cytoplasmic membranes. x 30,000.

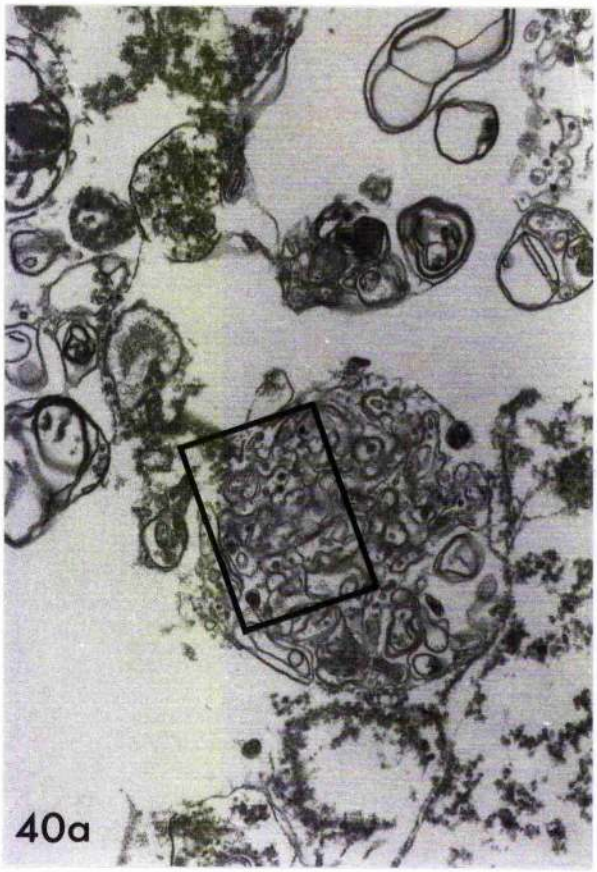
Fig 40b. A higher magnification of the area outlined in Figure 40a illustrates that some of the picornavirus-like particles are closely surrounded by membrane. x 150,000.



39a



39b



40a

40b

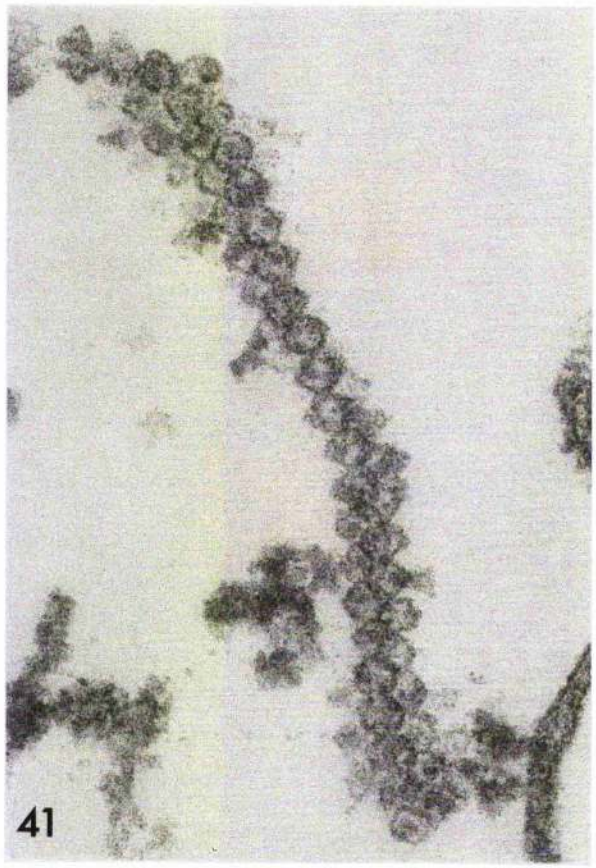
Figures 41 to 44 are of spontaneously detached cells from FRhK-4 culture (pass 81), fixed in suspension, 107 (Figs 41 & 42) and 128 (Figs 43 & 44) days after inoculation with HAV.

Fig 41. A chain of roughly spherical particles, vaguely reminiscent of R-type particles, lies in the cytoplasm of a degenerate cell. Where the plane of section is favourable small, sometimes eccentric, core-like structures can be seen within the particles. x 150,000.

Fig 42. The approximately spherical nature of the particles with their small, core-like structures is further illustrated in these arrays. x 150,000.

Fig 43. The small, irregular, core-like structures in some of these particles are more clearly visible than those in Figures 41 and 42. In addition, there is some indication of structures resembling the radial spokes of R-type particles. x 150,000.

Fig 44. Although the plane of section has grazed the surface of many of the particles in this field, small, core-like structures can be seen within those particles that have been favourably sectioned. x 150,000.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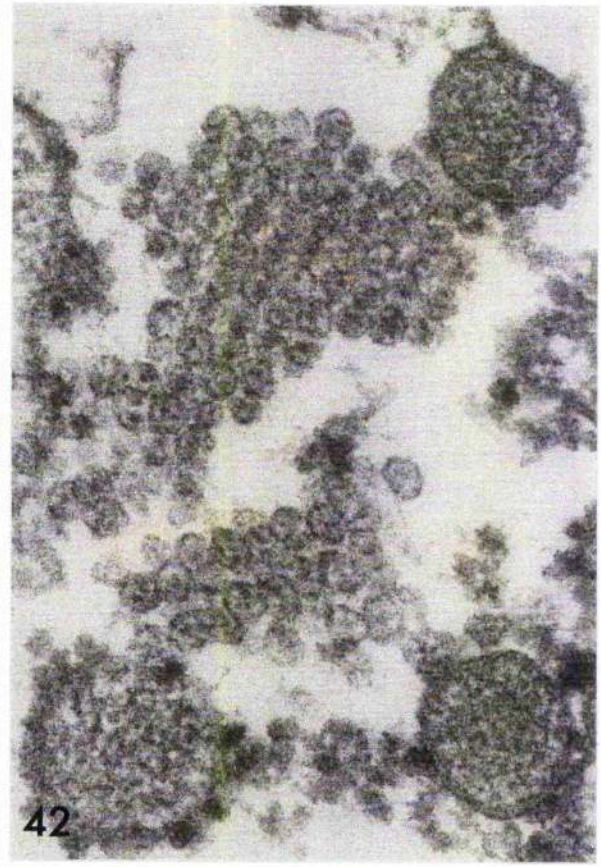
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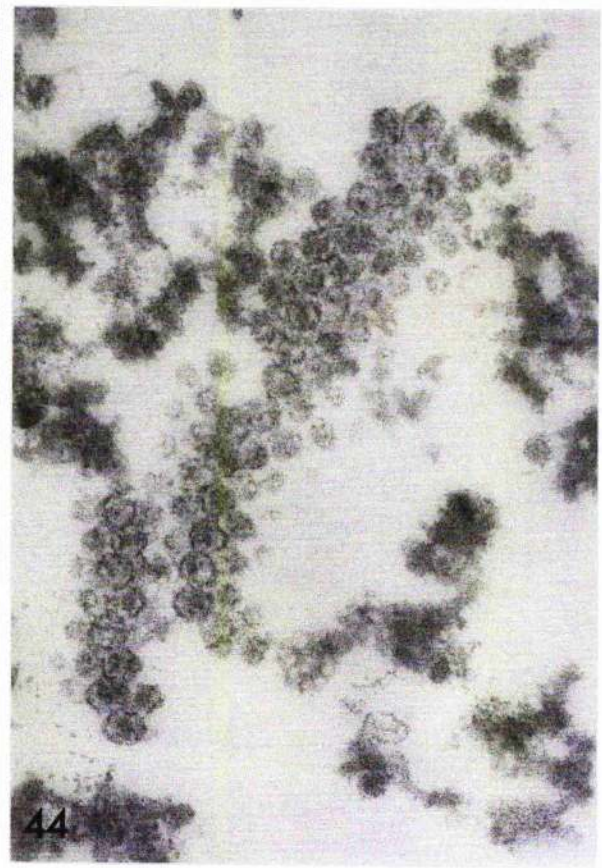
43


44

Figures 45 to 114 are electron micrographs of preparations negatively stained with phosphotungstic acid.

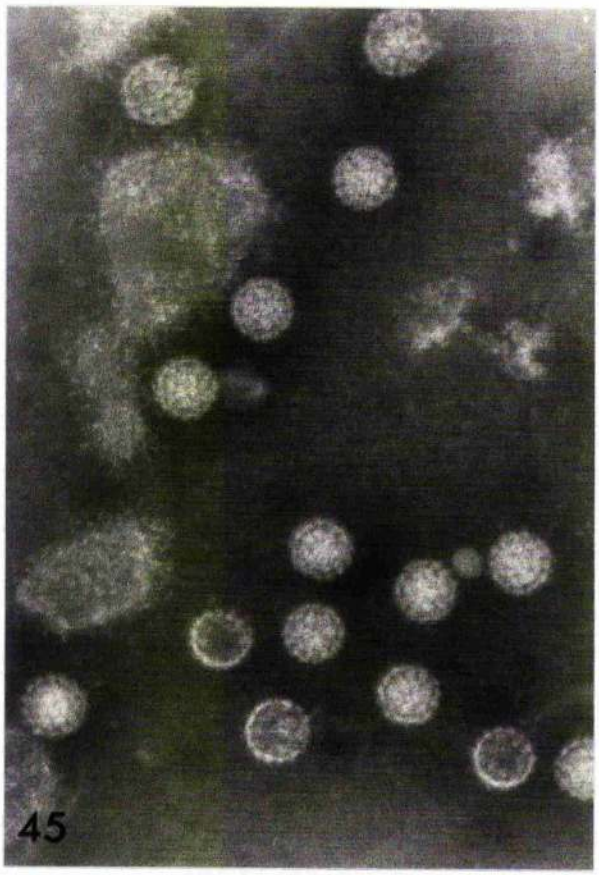
Figures 45 to 48 are of a high speed pellet of culture fluid from Vero cells inoculated with SV40; medium contained 2% newborn bovine serum.

Fig 45. Several polyomavirions, three of which are attached to fringed vacuoles, and a small, round, featureless particle are present. x 200,000.

Fig 46. Two clumps of polyomavirions and one "mini" polyomavirus particle can be seen. x 200,000.

Fig 47. Polyomavirions, free polyomavirus capsomers (arrow) and amorphous substance are illustrated. There are fine strands between two of the virus particles in the lower right part of the field. x 200,000.

Fig 48. In addition to polyomavirions and "mini" polyomavirus particles, there is fine amorphous substance in which a few capsomers (arrow) can be observed. x 200,000.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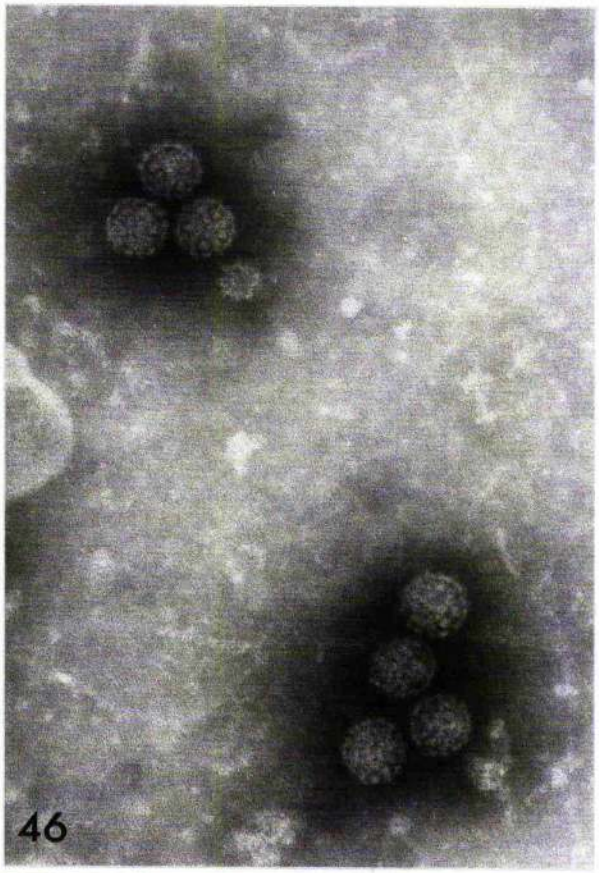
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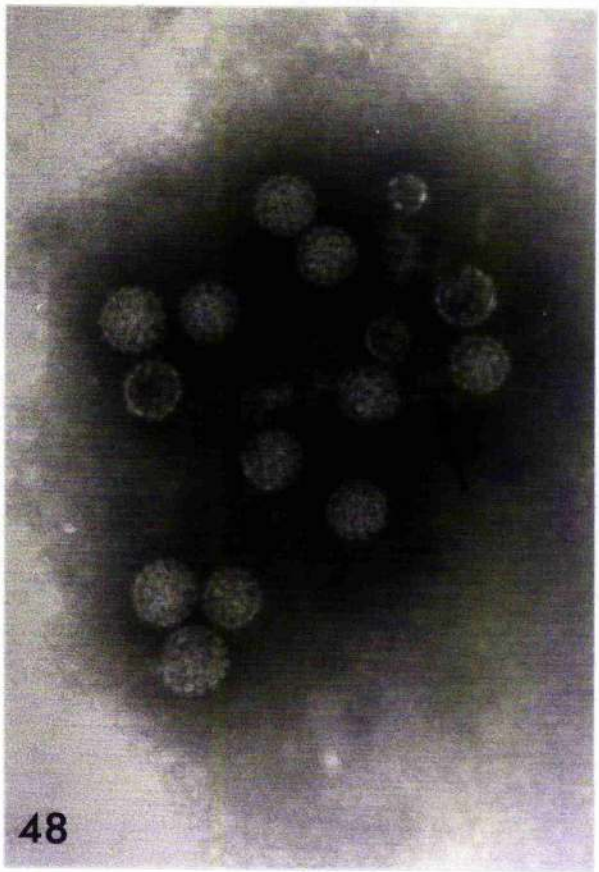

48

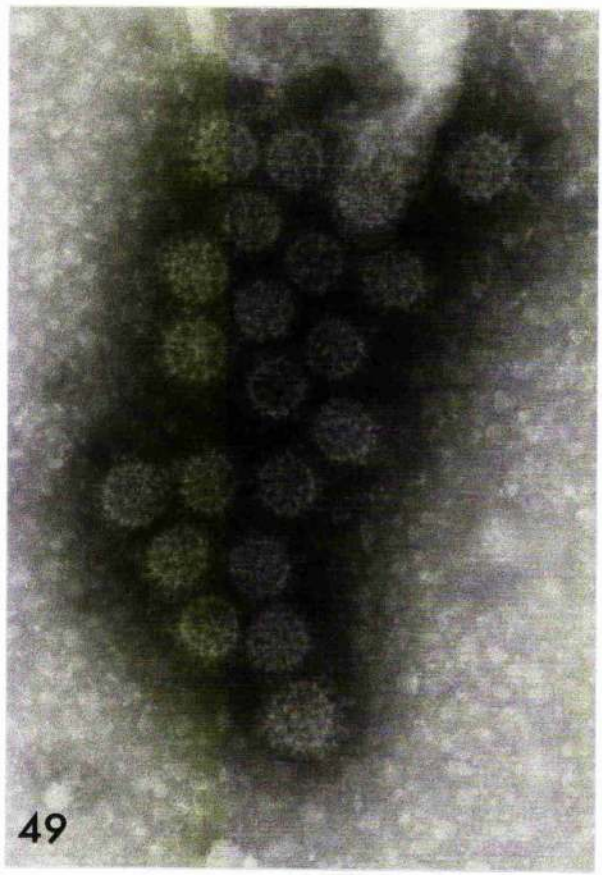
Figures 49 to 52 are of IEM reactions with SV40 and polyoma virus.

Fig 49. IEM: SV40 + calf SV40 antiserum, 1/25. Dense antibody surrounds and links the polyomavirus particles in a clump. x 200,000.

Fig 50. IEM: SV40 + calf SV40 antiserum, 1/25. Densely antibody-coated polyomavirions are attached to a vacuole into which phosphotungstic acid has failed to penetrate. The three circular projections might represent polyomavirus particles within the vacuole. x 200,000.

Fig 51. IEM: polyoma virus + mouse polyoma virus antiserum, 1/500. Polyoma virus particles are clumped by very small amounts of antibody. x 200,000.

Fig 52. IEM: polyoma virus + mouse polyoma virus antiserum, 1/500. Very small amounts of antibody clump the polyoma virions and a "giant" polyoma virus particle. x 200,000.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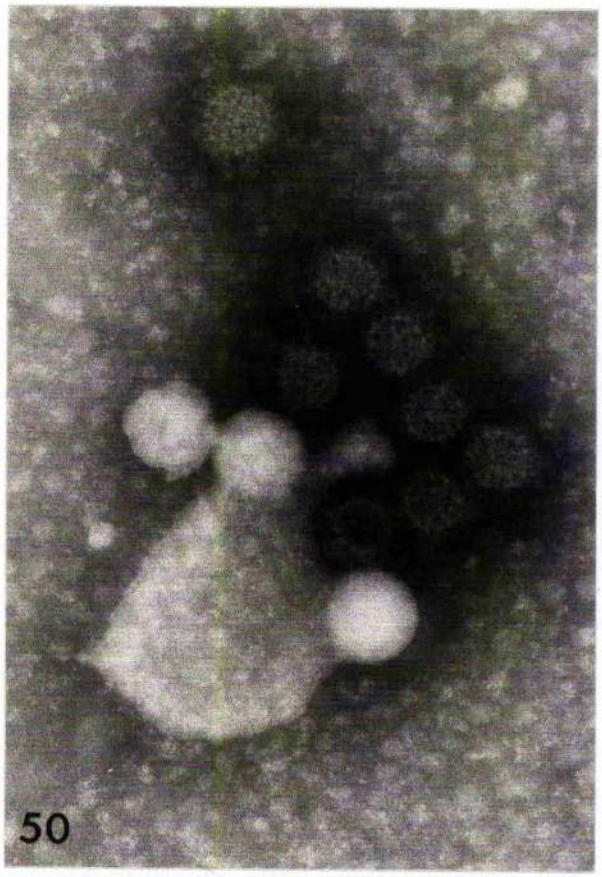
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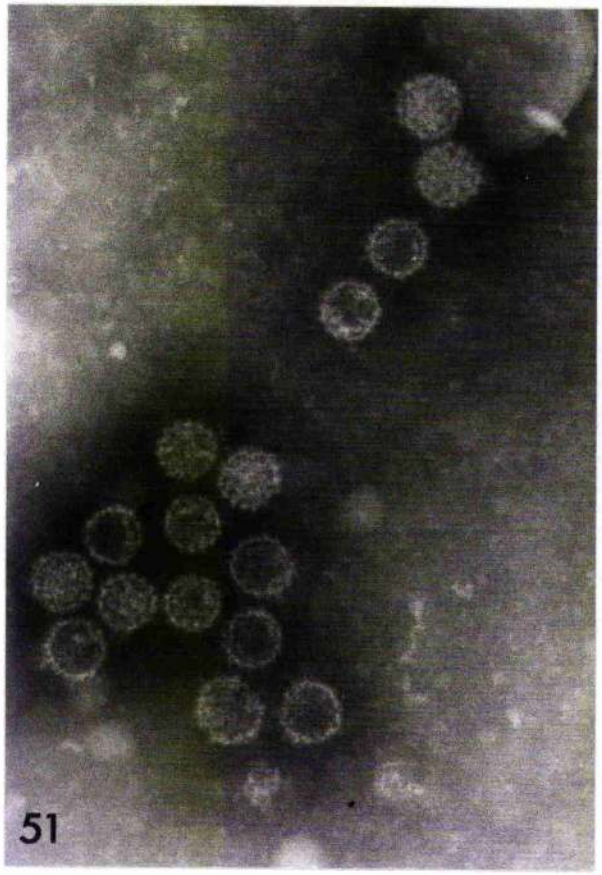
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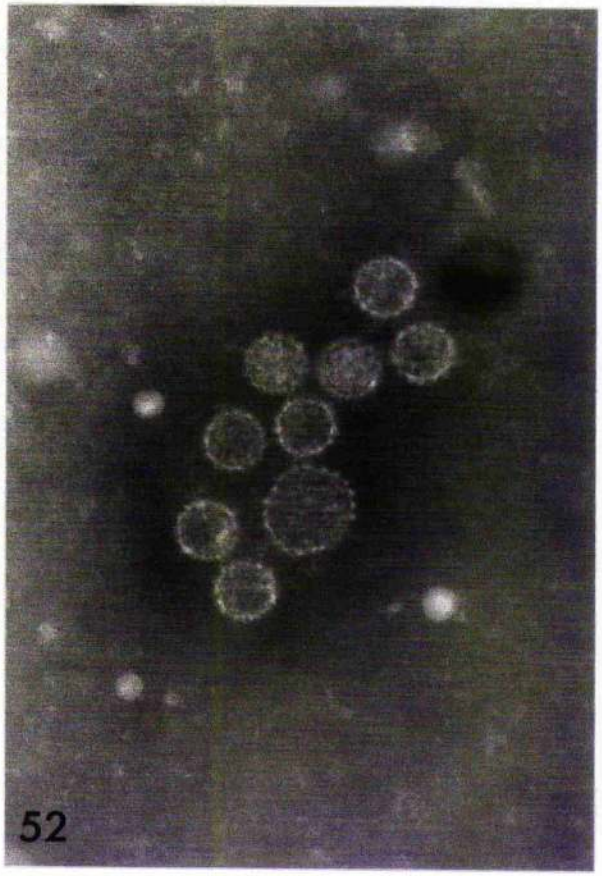

52

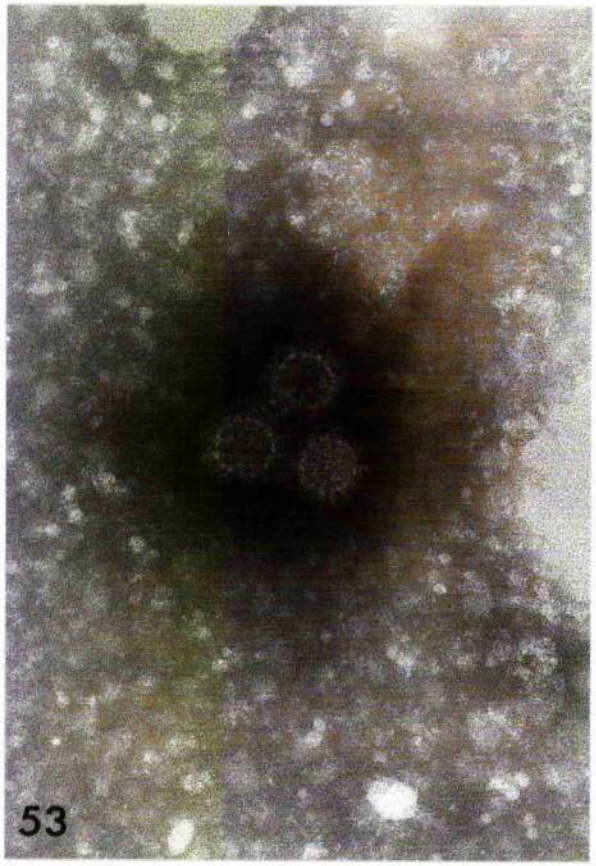
Figures 53 to 56 are of IEM reactions with FRK4V from a high speed pellet of clarified culture fluid from control, uninoculated FRhK-4 cells after incubation for 22 days; Iscove's serum-free medium.

Fig 53. IEM: FRK4V + newborn bovine serum 17/8, 1/10. A dense layer of antibody surrounds and links these three polyomavirus particles close to which is some amorphous substance. x 200,000.

Fig 54. IEM: FRK4V + rabbit STMV antiserum 81/239, 1/5. The polyomavirus particles in this clump are surrounded by a particularly dense layer of antibody. Amorphous substance is attached to the virus-antibody aggregate. x 200,000.

Fig 55. IEM: FRK4V + rabbit STMV antiserum 75/176, 1/5. A dense layer of antibody clumps three polyomavirions. x 200,000.

Fig 56. IEM: FRK4V + rabbit STMV antiserum 75/176, 1/5. Near to a clump of five densely antibody-coated polyomavirions there is some amorphous substance. x 200,000.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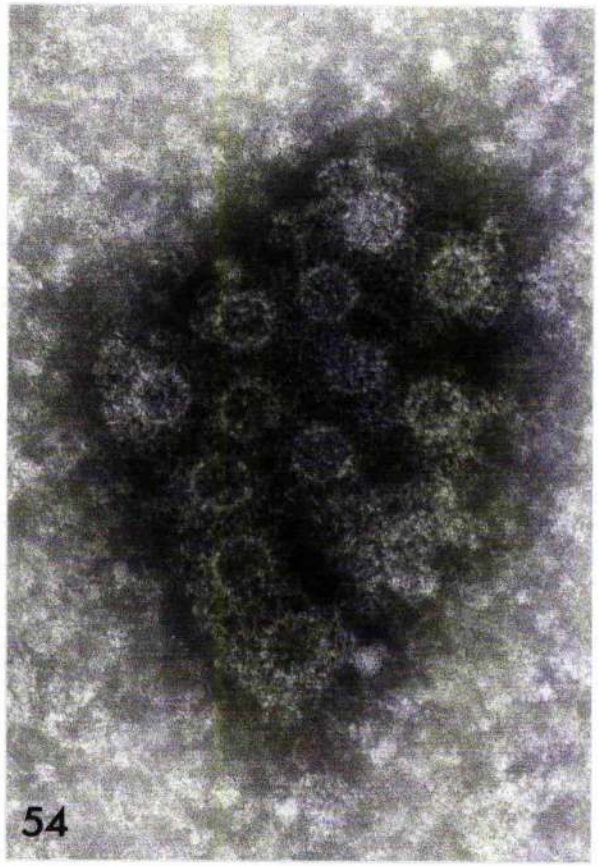
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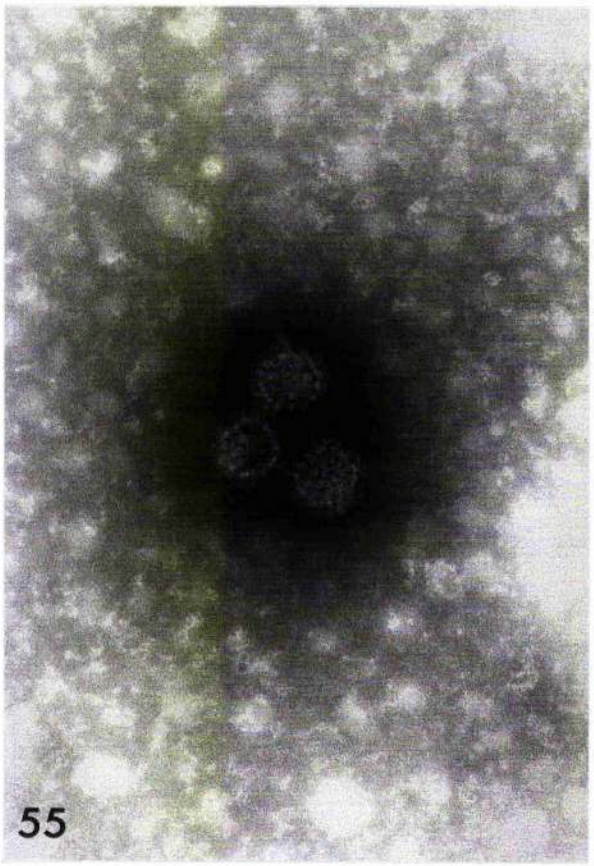
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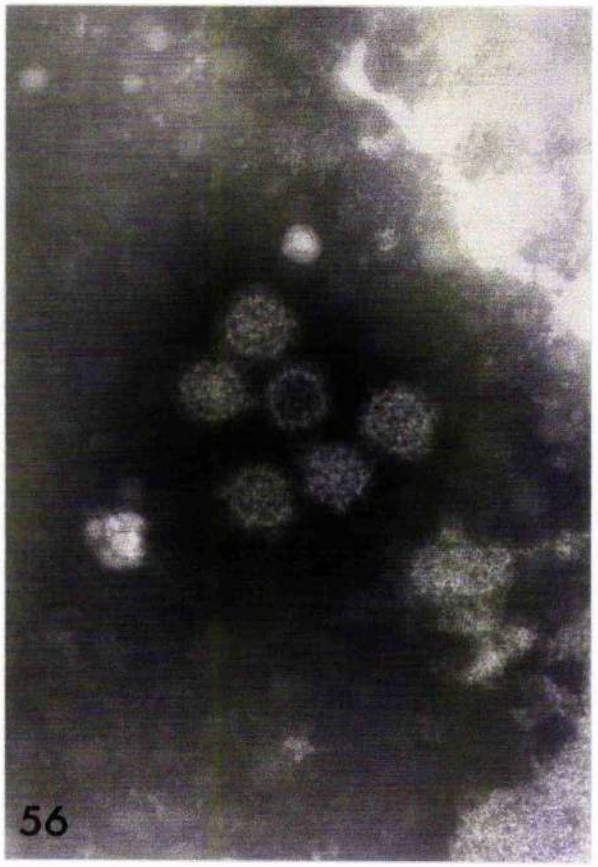
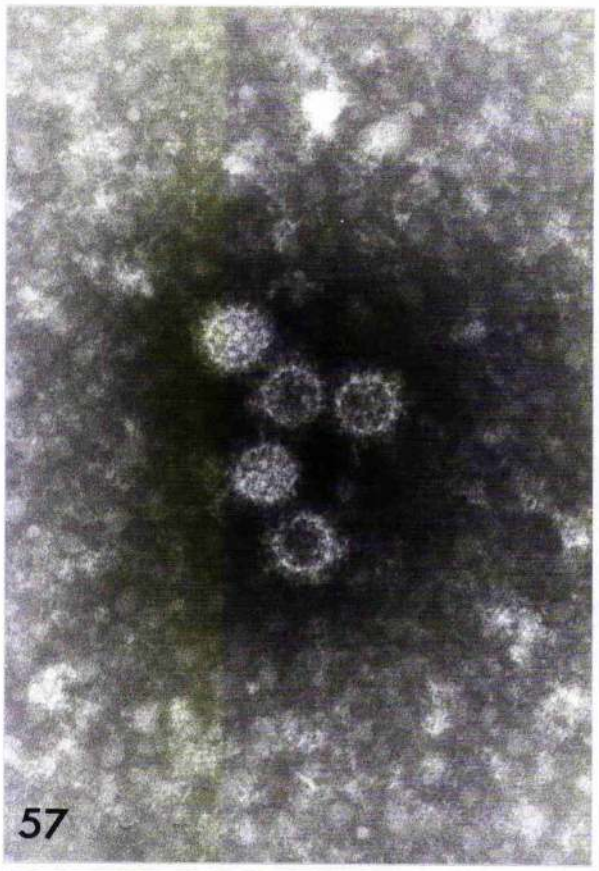

56

Figures 57 to 59 are of IEM reactions with FRK6V. Virus from a high speed pellet of culture fluid from uninoculated FRhK-6 culture after incubation for six days with Iscove's serum-free medium (Figs 57 & 58) and spontaneously detached CK17 cells 39 days after inoculation with FRK6V (Fig 59).

Fig 57. IEM: FRK6V + newborn bovine serum 17/8, 1/10. Five clumped polyomavirions are densely coated with antibody. x 200,000.

Fig 58. IEM: FRK6V + newborn bovine serum 22/6, 1/10. Dense antibody surrounds and links three polyomavirions. x 200,000.

Fig 59. IEM: FRK6V + rabbit STMV antiserum 75/176, 1/10. Compare the appearance of the densely antibody-coated polyomavirions and filaments in the large aggregate with that of the membrane-associated polyomavirions in the smaller group, towards the top left of the field, which are not coated with antibody. x 60,000.



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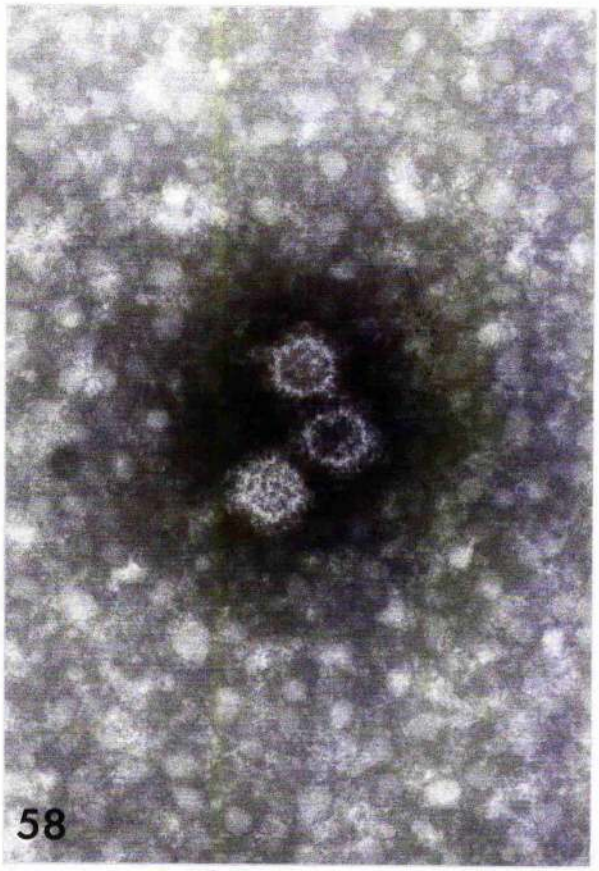

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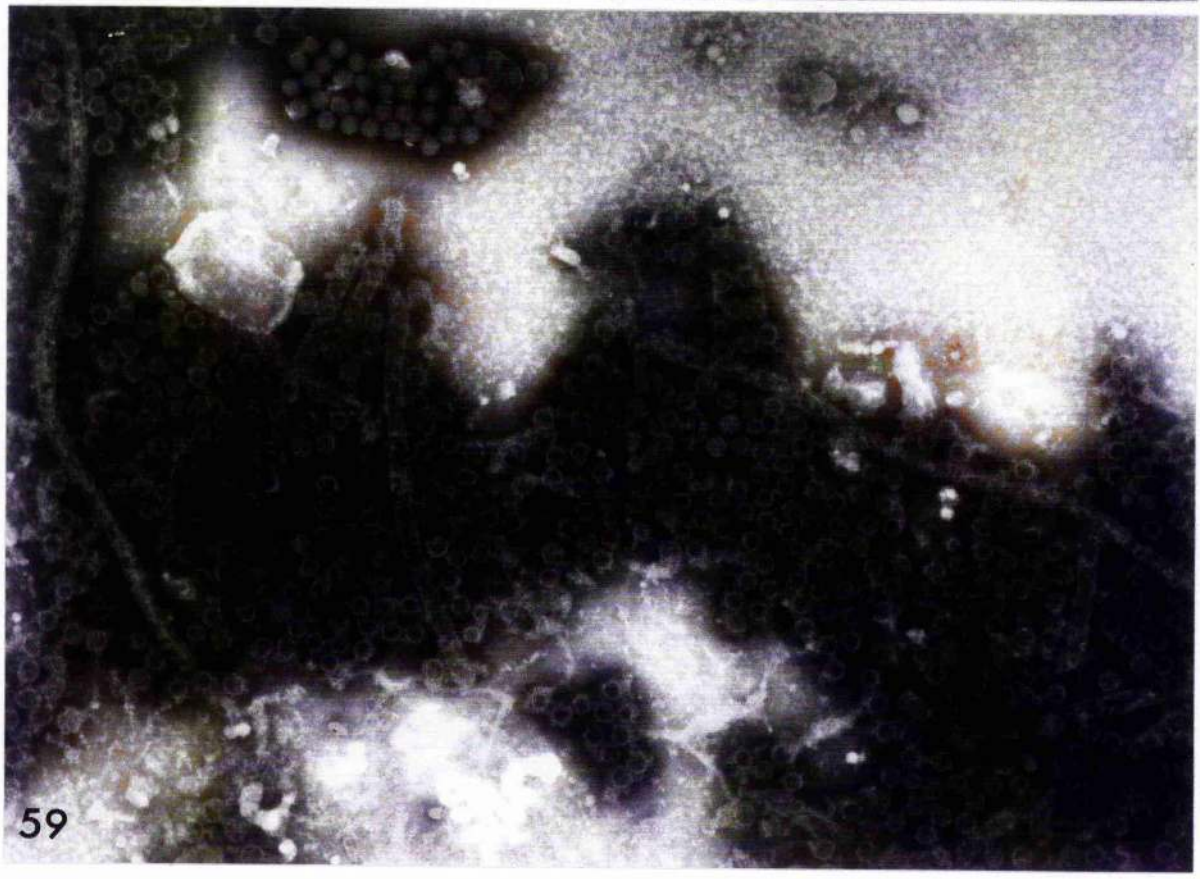


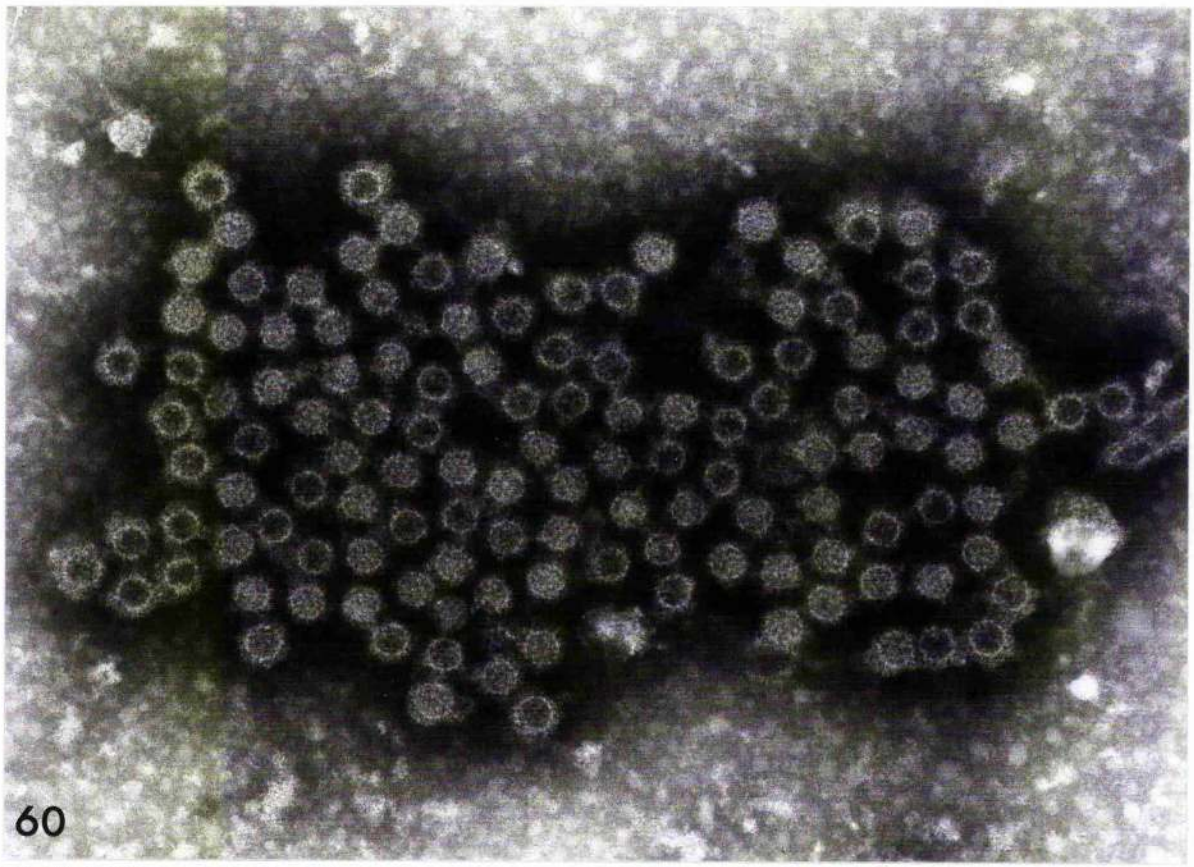
59

Figures 60 to 62 are of IEM reactions with WRSV. Virus from a high speed pellet of culture fluid from CK8 cells seven days after inoculation with WRSV; medium contained 2% equine serum.

Fig 60. IEM: WRSV + rabbit STMV antiserum 81/239, 1/20. Polyomavirions are densely coated with antibody in this large clump. x 100,000.

Fig 61. IEM: WRSV + rabbit STMV antiserum 81/239, 1/10. Numerous polyomavirions, including a "mini mini" particle, are clumped by dense antibody coating. x 200,000.

Fig 62. IEM: WRSV + rabbit STMV antiserum 81/239, 1/10. A vacuole, similar to that in Figure 50, is illustrated. Phosphotungstic acid has penetrated the membrane of two of the projections to reveal polyomavirions which do not have antibody attached; the stain has failed to penetrate the remaining three protrusions which might also contain polyomavirus particles. x 200,000.



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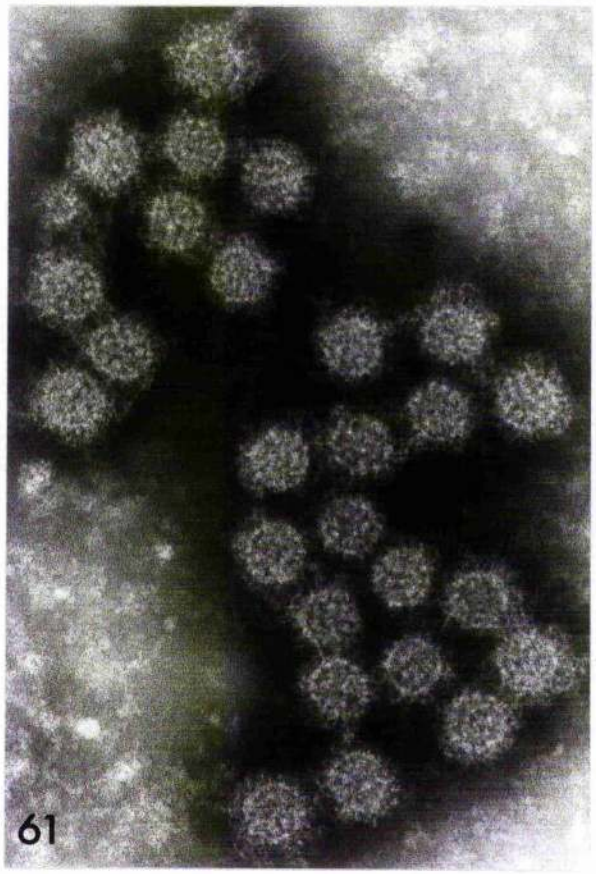

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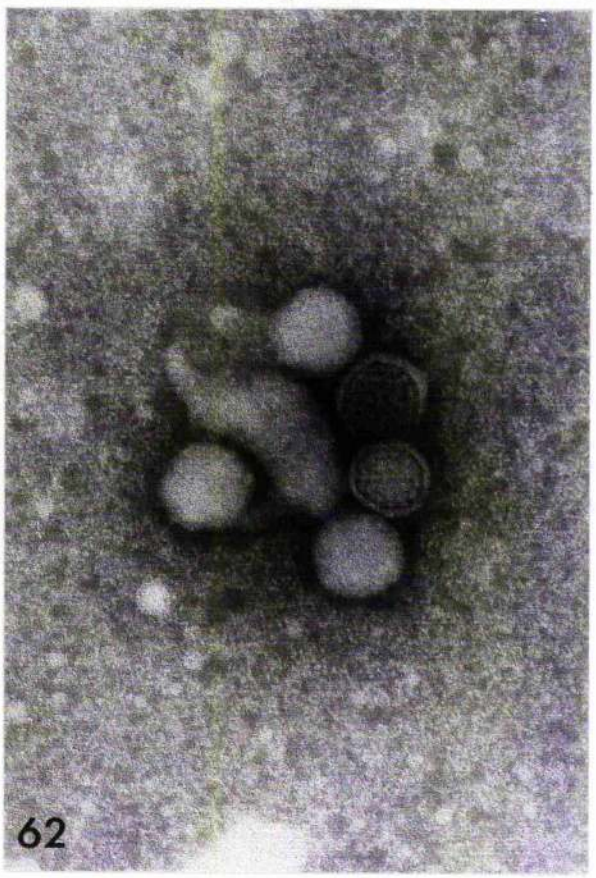


62

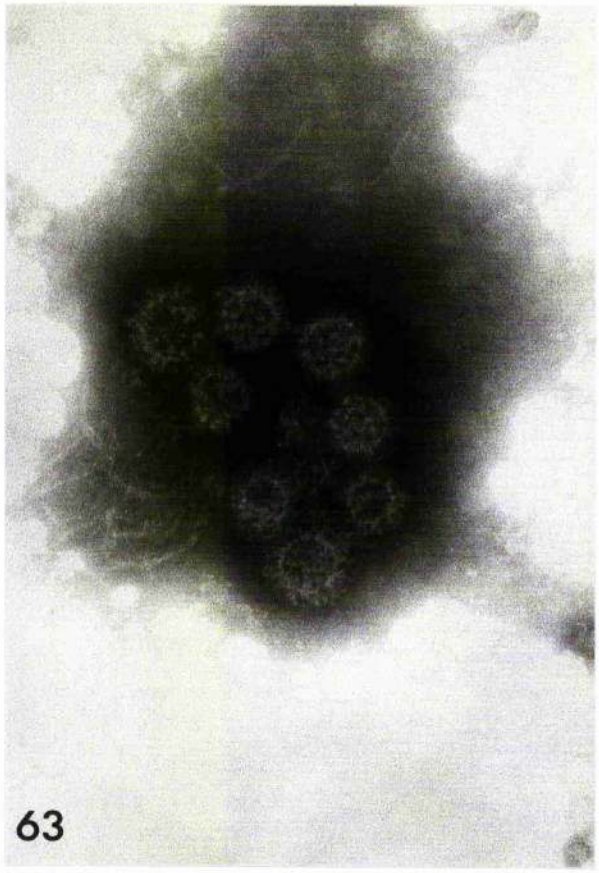
Figures 63 to 66 are of homogenized preparations of gel lines formed by counter-immunoelectrophoresis (CIE) with FRK4V from spontaneously detached cells from control, uninoculated FRhK-4 cultures.

Fig 63. CIE: FRK4V + RDE-treated newborn bovine serum 22/6. Fine strands of agarose lie near this clump of densely antibody-coated polyomavirus particles. x 200,000.

Fig 64. CIE: FRK4V + serum from a calf aged one week. Six polyomavirions are clumped by dense antibody. x 200,000.

Fig 65. CIE: FRK4V + serum from a calf aged between two and eight months. Dense antibody surrounds and clumps five polyomavirions. x 200,000.

Fig 66. CIE: FRK4V + rabbit FRKV antiserum. Evidence of antibody cannot be seen on this piece of polyomavirus filament from the ends of which component capsomers are separating. x 200,000.



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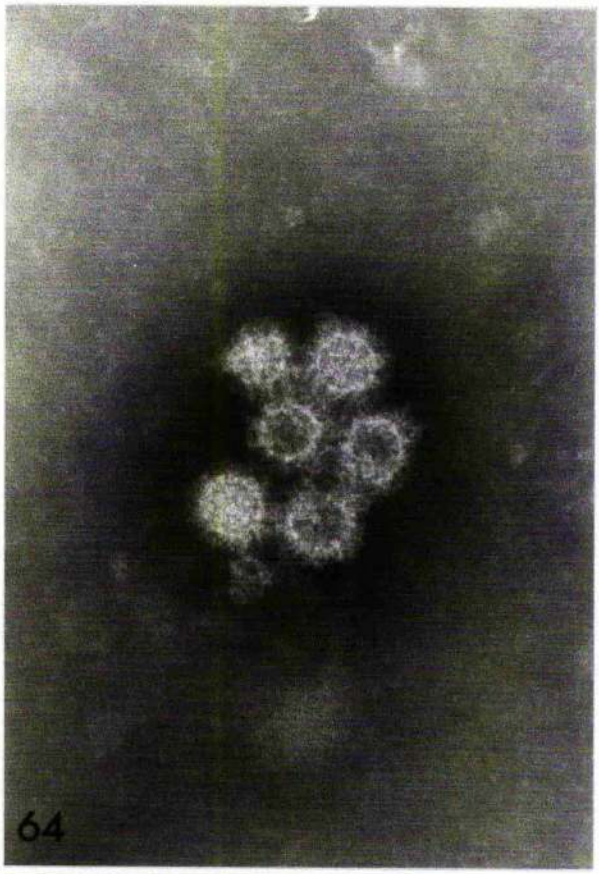

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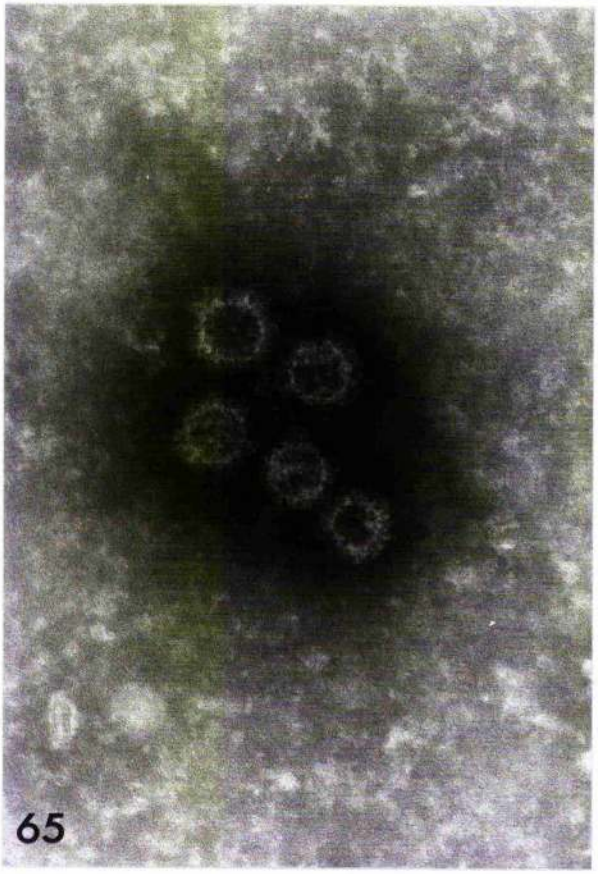

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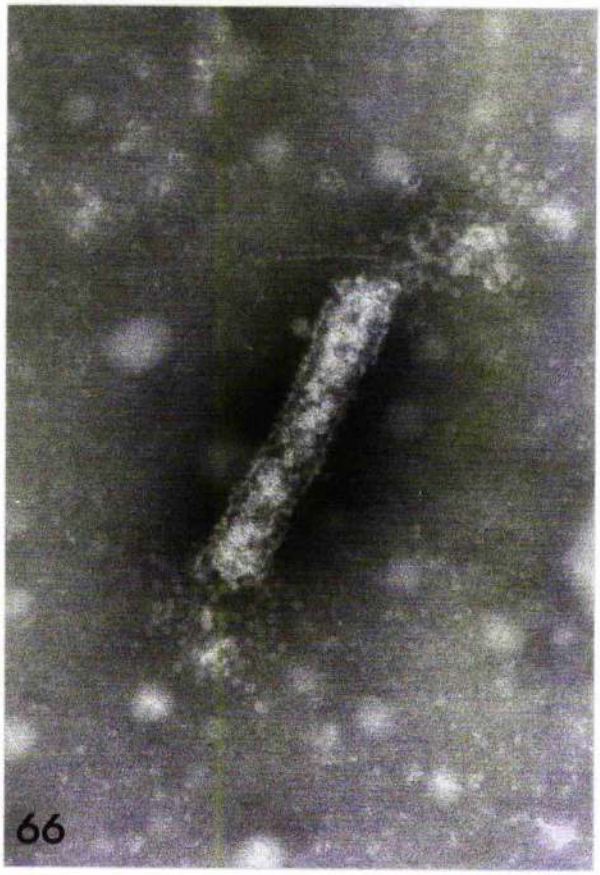

66

Figures 67 and 68 are of a high speed pellet of culture fluid from uninoculated LLC-MK2 cells (pass 149) after incubation for 56 days; medium contained 2% newborn bovine serum.

Fig 67. Two small, round, featureless particles are associated with two polyomavirions with little or no antibody; the remaining two polyomavirions have moderate amounts of antibody attached. x 200,000.

Fig 68. Close to two moderately antibody-coated polyomavirions there is a plume of slightly electron-translucent substance. x 200,000.

Figures 69 and 70 are of IEM reactions with LLC-MK2V, the polyomavirus illustrated above.

Fig 69. IEM: LLC-MK2V + goat anti-bovine IgG, 1/20. To this clump of three very densely antibody-coated polyomavirions is attached one virus particle that has little antibody on its surface. x 200,000.

Fig 70. IEM: LLC-MK2V + goat anti-bovine IgG, 1/20. A polyomavirus particle with practically no antibody is attached to two polyomavirions and a "mini" particle which are very densely antibody-coated. x 200,000.


67


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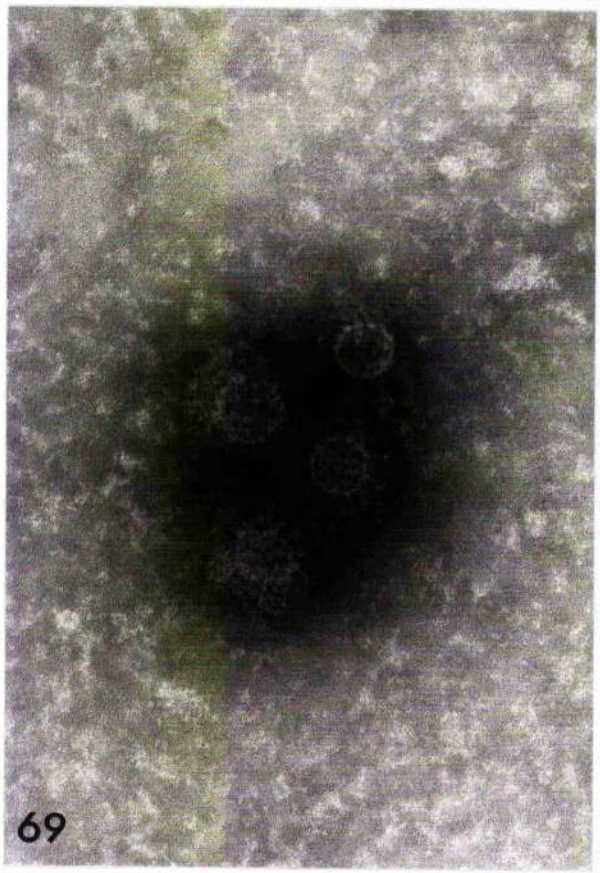
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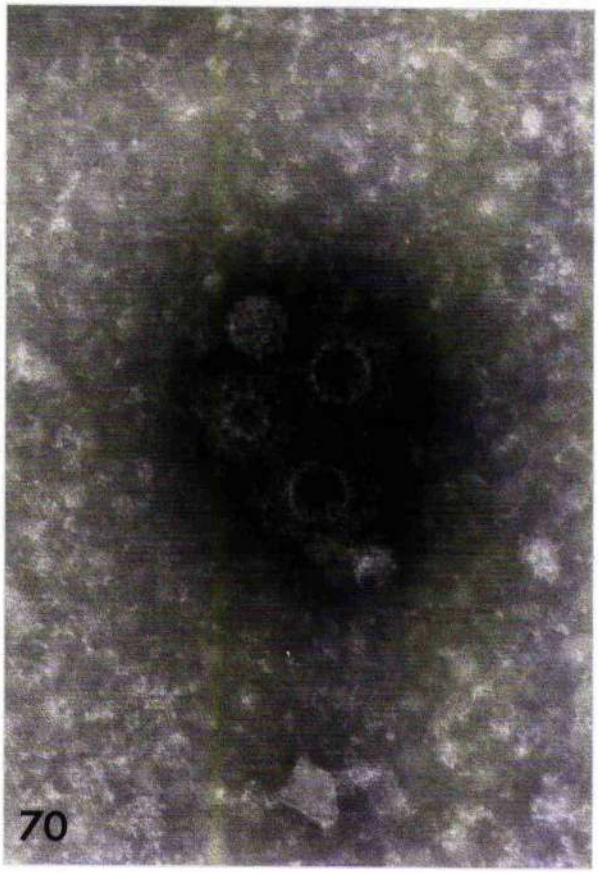
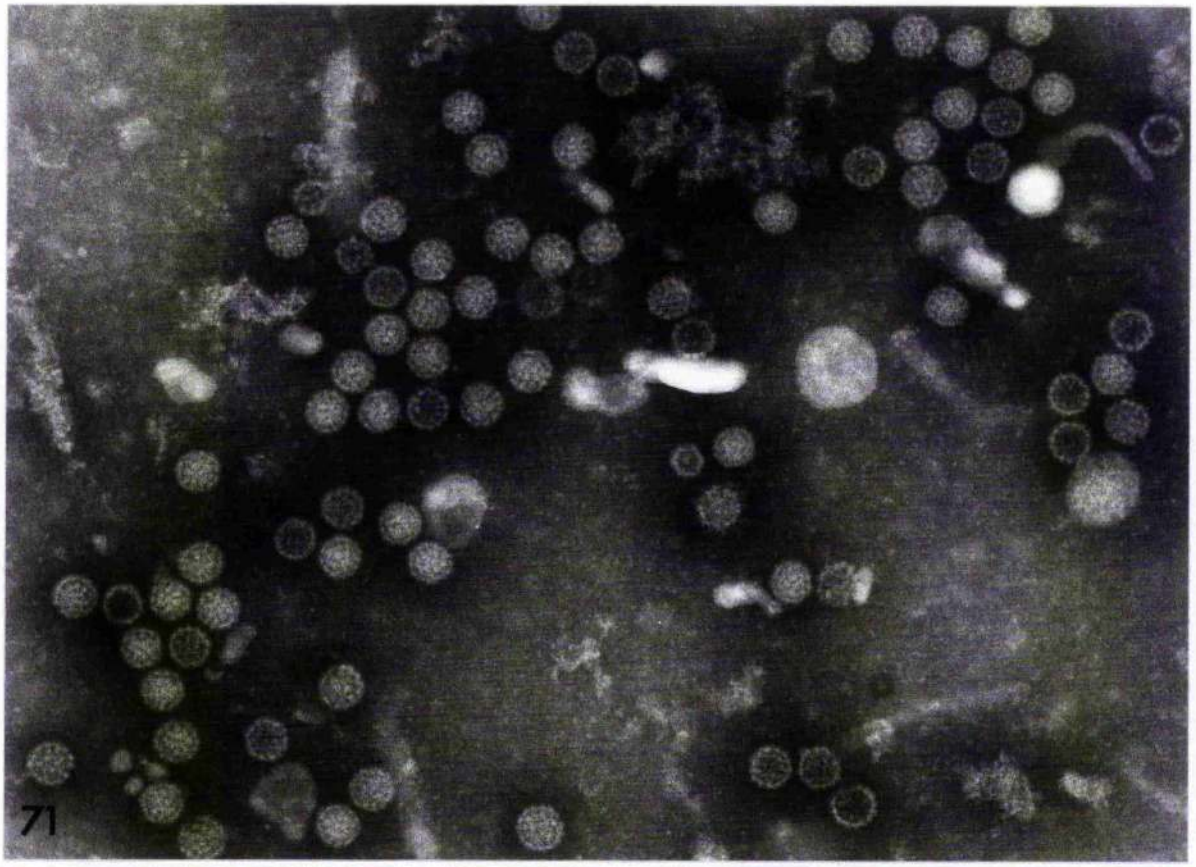

70

Figures 71 to 73 are of culture fluids from uninoculated CK cells; media contained 2% fetal bovine serum U720201D. High speed pellets of culture fluids (Figs 71 & 72) and of clarified culture fluid (Fig 73).

Fig 71. CK12 culture after incubation for 19 weeks. A single polyomavirus particle with a dense layer of antibody is surrounded by large numbers of polyomavirions without antibody. Vacuoles and amorphous substance are also present. x 150,000.

Fig 72. CK22 culture after incubation for 21 weeks. Two "mini mini" polyomavirus particles are associated with amorphous substance and three more lie beside part of a polyomavirus filament. x 200,000.

Fig 73. CK27 culture after incubation for 18 weeks. Small, round or oval, featureless particles are attached to polyomavirions, several of which are damaged. x 200,000.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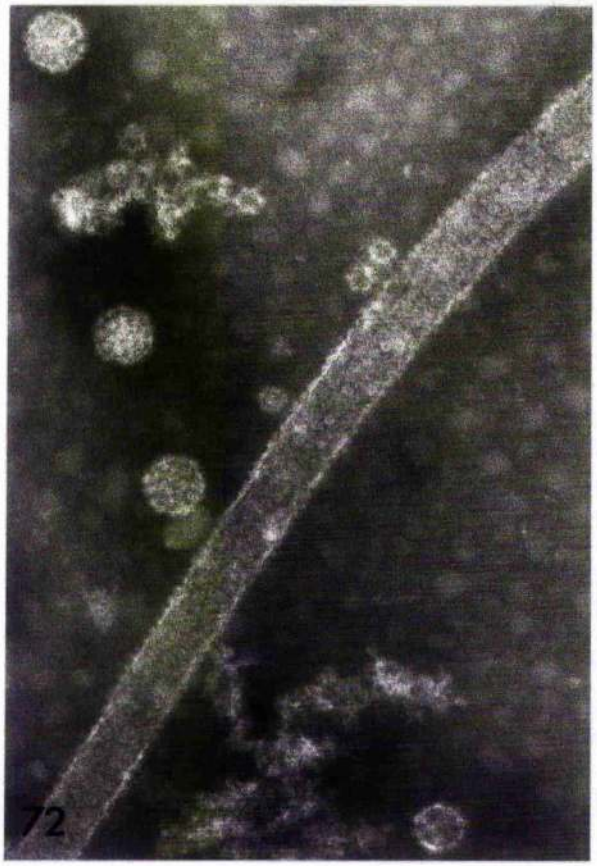
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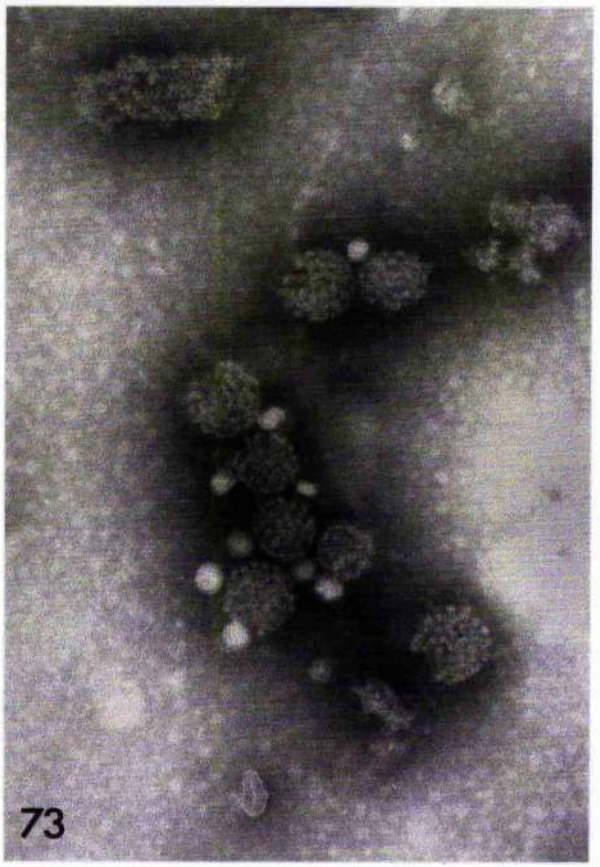

73

Figures 74 and 75 are of high speed pellets of culture fluids from two batches of uninoculated CK29 cells after incubation for ten weeks.

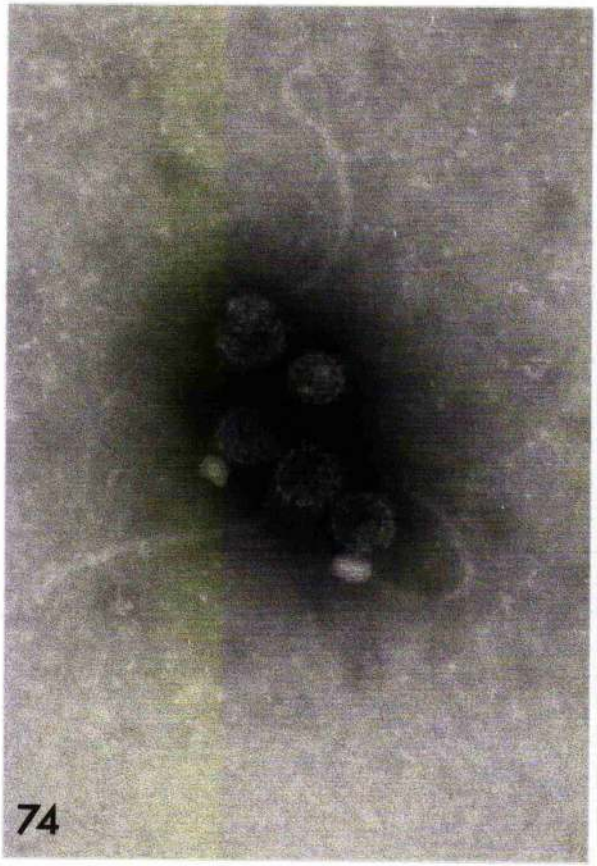
Fig 74. CK29/1 culture; medium contained 2% gamma-irradiated fetal bovine serum. Three trails of slightly electron-translucent substance lie close to a clump of "clean" polyomavirions, polyomavirus particles with moderate amounts of antibody and two small, oval, featureless particles. x 200,000.

Fig 75. CK29/2 culture; medium contained 2% equine serum. Evidence of antibody cannot be found on either the polyomavirions or the narrow polyomavirus filaments. A few capsomers are associated with amorphous substance (arrow). x 200,000.

Figures 76 and 77 are of IEM reactions with the two CK29 polyomaviruses illustrated above.

Fig 76. IEM: CK29/1V (from CK29/1) + rabbit STMV antiserum 75/176, 1/50. The polyomavirions are clumped by moderate amounts of antibody. x 200,000.

Fig 77. IEM: CK29/2V (from CK29/2) + rabbit STMV antiserum 75/176, 1/50. There are small amounts of antibody between the polyomavirions in this clump. x 200,000.



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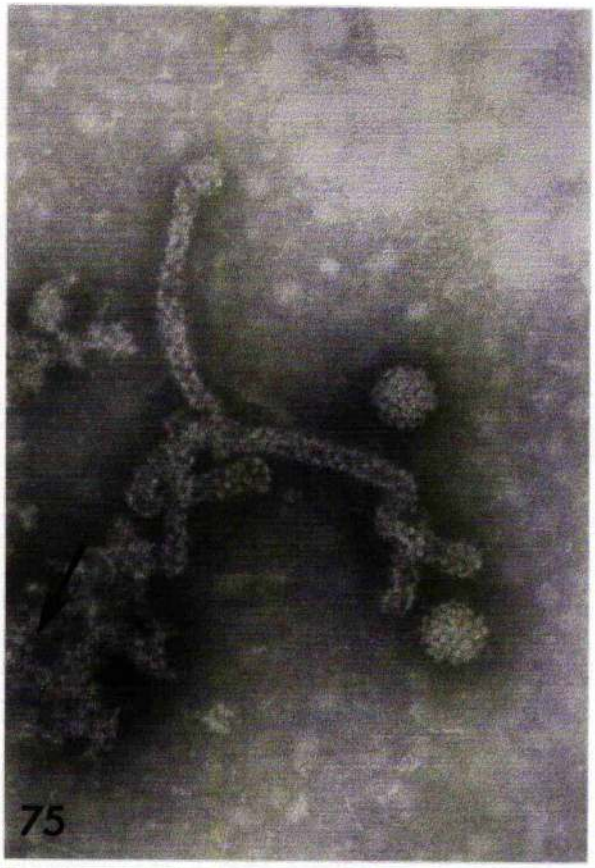

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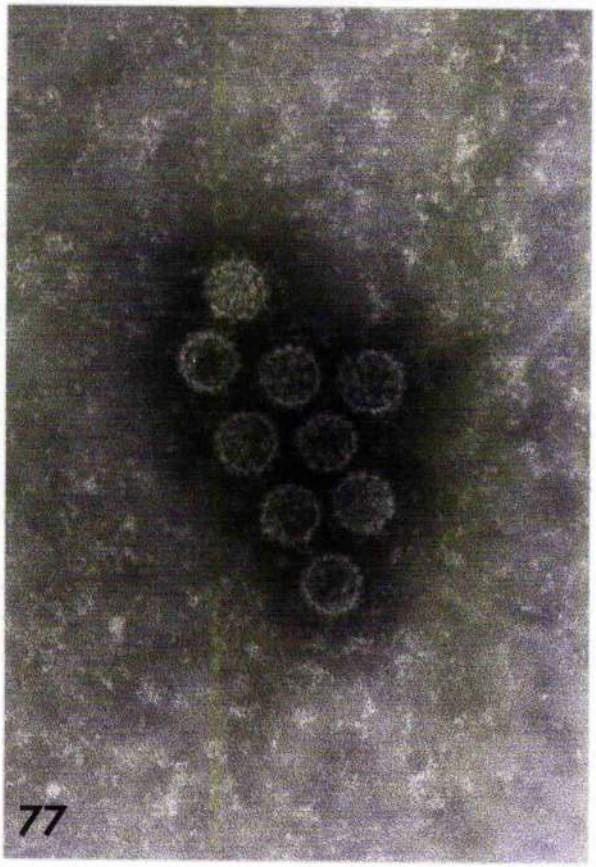


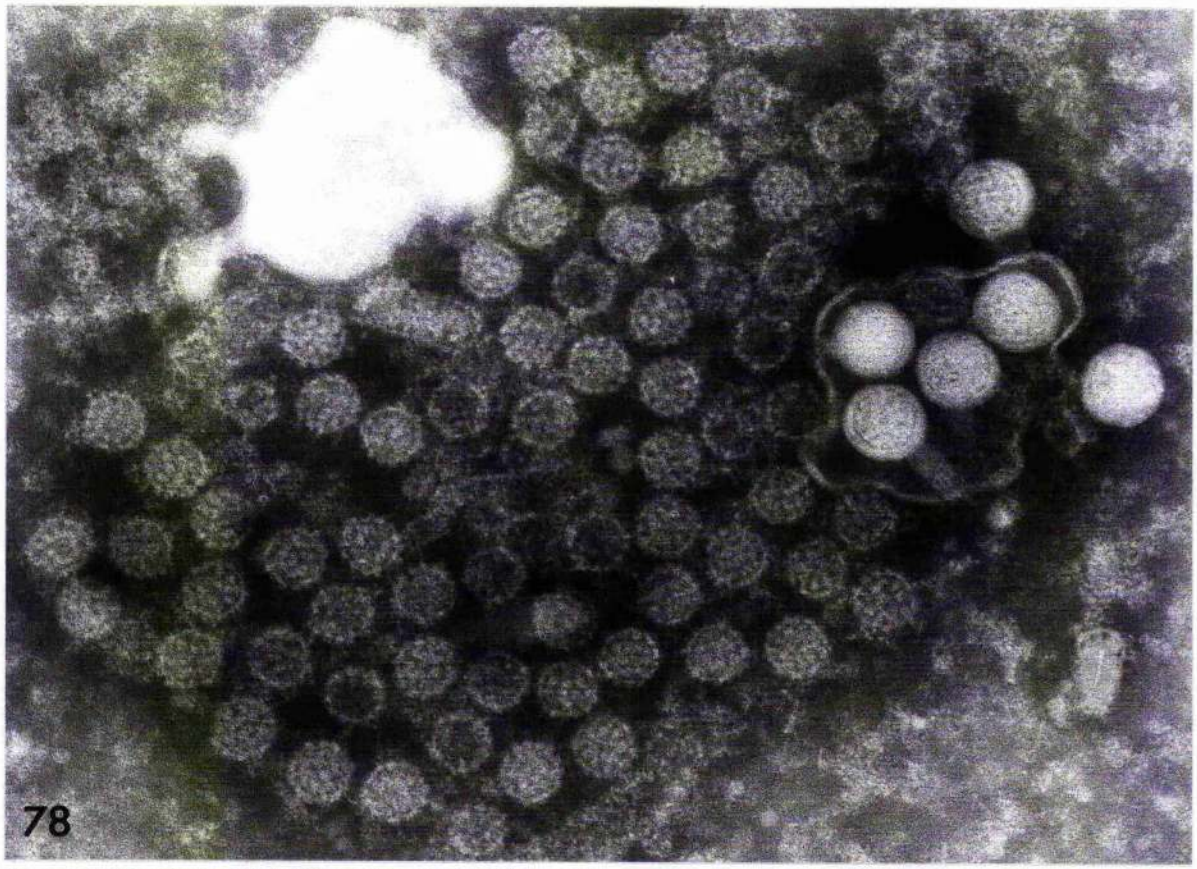
77

Figures 78 to 80 are of IEM reactions with the polyomavirus isolates from high speed pellets of culture fluids from uninoculated CK8, CK9 and CK10 cells; media contained 2% fetal bovine serum U720201D.

Fig 78. IEM: CK8V + rabbit STMV antiserum 81/239, 1/10. Moderate amounts of antibody clump the polyomavirions in this large aggregate. Differing degrees of penetration of the vacuole membranes by phosphotungstic acid has revealed the morphology of the entrapped polyomavirions to various extents. x 200,000.

Fig 79. IEM: CK9V + newborn bovine serum 21/6, 1/10. There are small amounts of antibody between these polyomavirus particles and a faint trail of slightly electron-translucent substance can also be seen. x 200,000.

Fig 80. IEM: CK10V + rabbit STMV antiserum 75/176, 1/10. Moderate amounts of antibody surround the polyomavirions in this clump which lies between two vacuoles. x 200,000.



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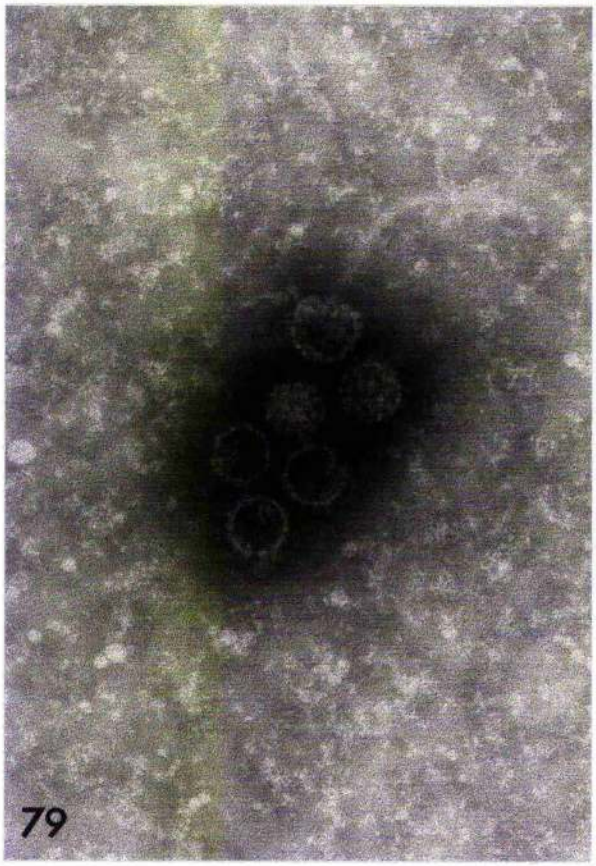

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Figures 81 and 82 are of IEM reactions with the polyomavirus from a high speed pellet of culture fluid from uninoculated CK12 cells; medium contained 2% fetal bovine serum U720201D.

Fig 81. IEM: CK12V + newborn bovine serum 21/6, 1/10. Antibody could not be identified on the surface of the polyomavirions in this preparation. x 200,000.

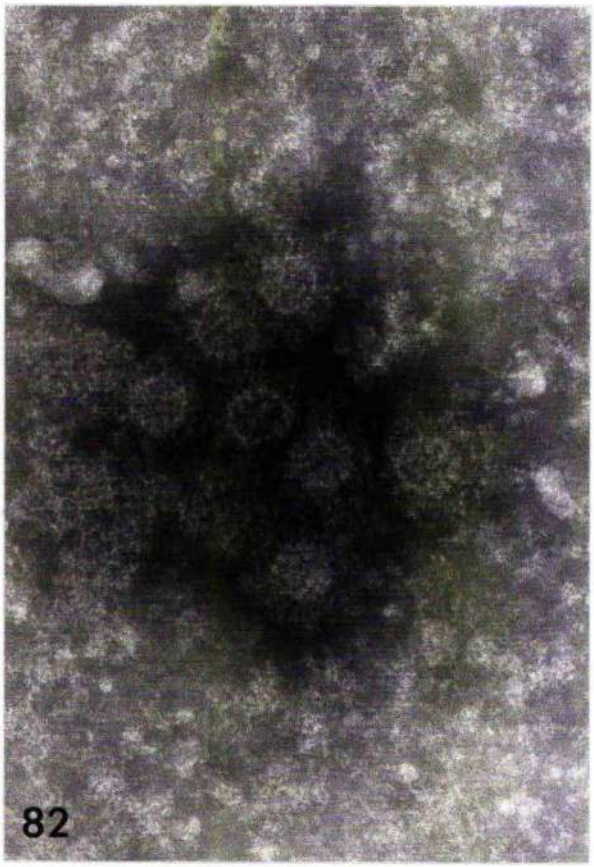
Fig 82. IEM: CK12V + rabbit STMV antiserum 81/239, 1/20. The antibody coating of the polyomavirions in this clump is particularly dense. x 200,000.

Figures 83 and 84 are of IEM reactions with CK29/1V.

Fig 83. IEM: CK29/1V + newborn bovine serum 21/6, 1/10. Dense antibody coating of the polyomavirions is illustrated. x 200,000.

Fig 84. IEM: CK29/1V + rabbit STMV antiserum 75/176, 1/20. Five polyomavirions in a clump and one single virus particle are all surrounded by dense antibody. x 200,000.


81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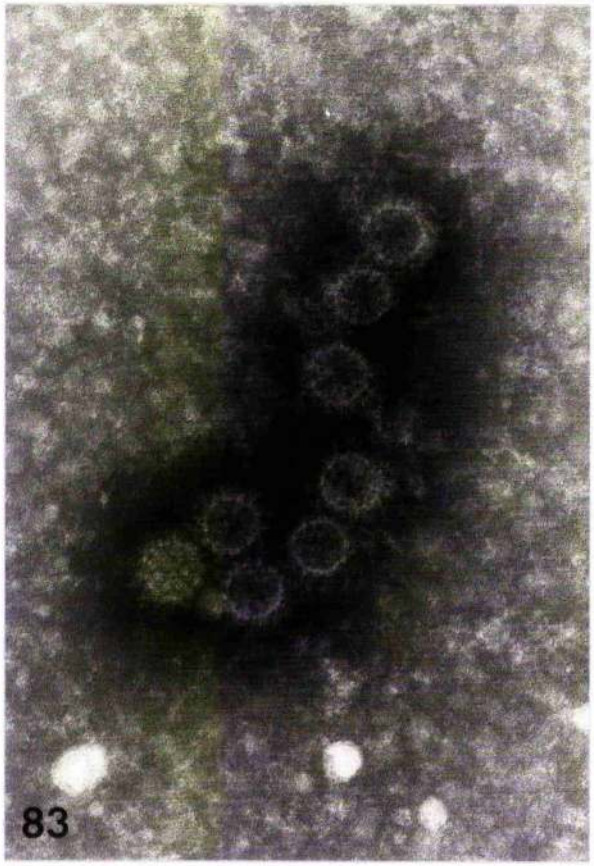
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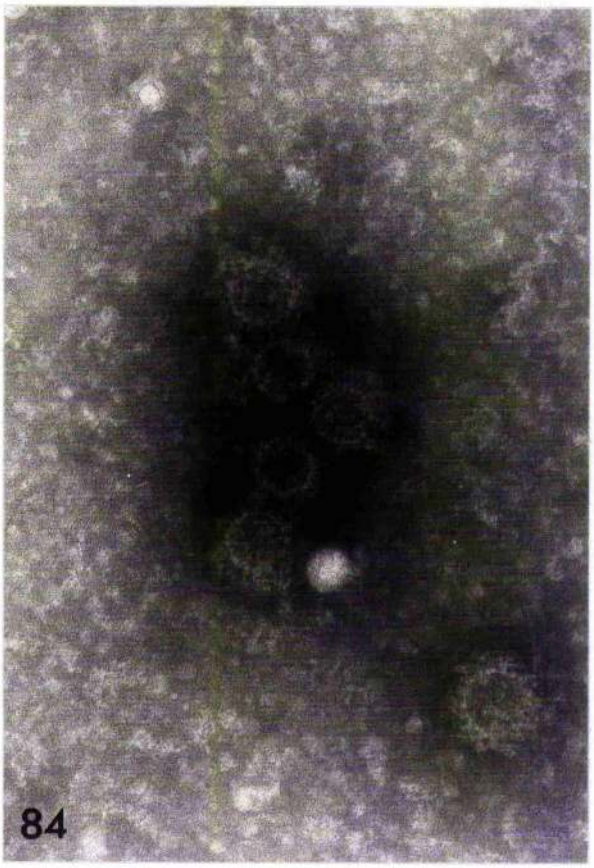

84

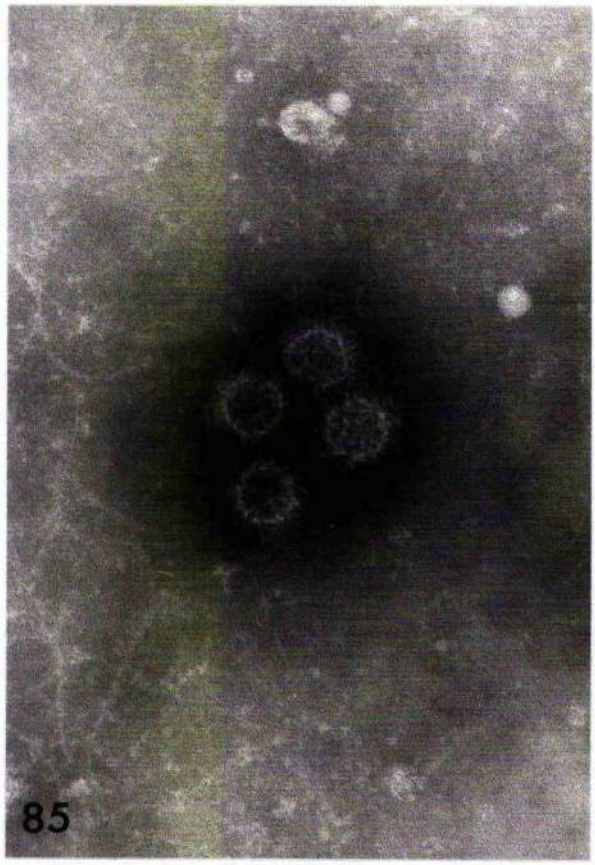
Figures 85 to 88 illustrate the use of antibody-coated grids (ACG). Control grids, or grids coated with goat anti-bovine (GAB) IgG, were floated on preparations of CK14V. Virus from a high speed pellet of clarified culture fluid from uninoculated CK14 cells after incubation for 17 weeks; medium contained 2% fetal bovine serum U720201D.

Fig 85. ACG: control grid (without GAB IgG) + CK14V. Four polyomavirions are clumped by dense antibody. x 200,000.

Fig 86. ACG: grid treated with GAB IgG, 1/100 + CK14V. Polyomavirions, a polyomavirus filament and "mini mini" particles are all surrounded by dense antibody. x 200,000.

Fig 87. ACG: grid treated with GAB IgG, 1/500 + CK14V. Three polyomavirions have dense antibody coating. x 200,000.

Fig 88. ACG: grid treated with GAB IgG, 1/1000 + CK14V. Dense antibody surrounds the polyomavirions and "mini" polyomavirus particles in this clump. x 200,000.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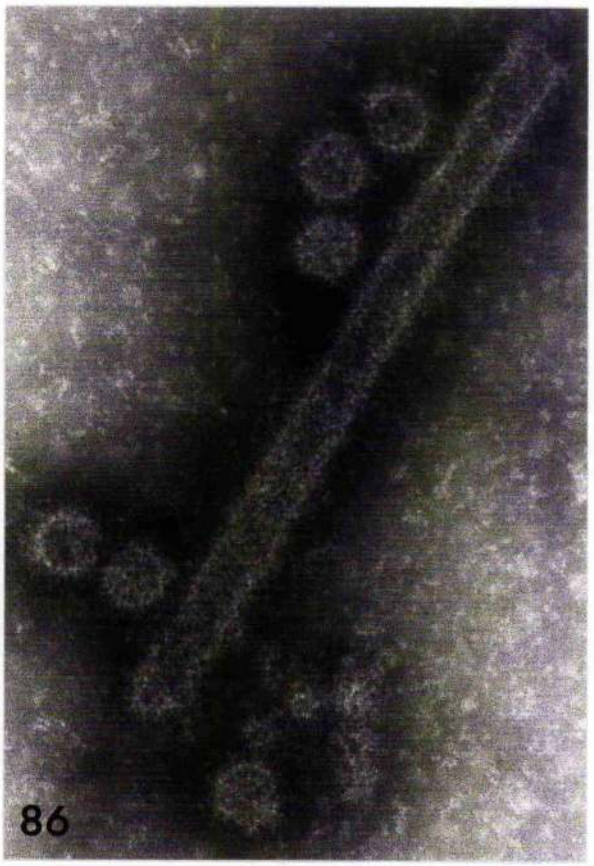
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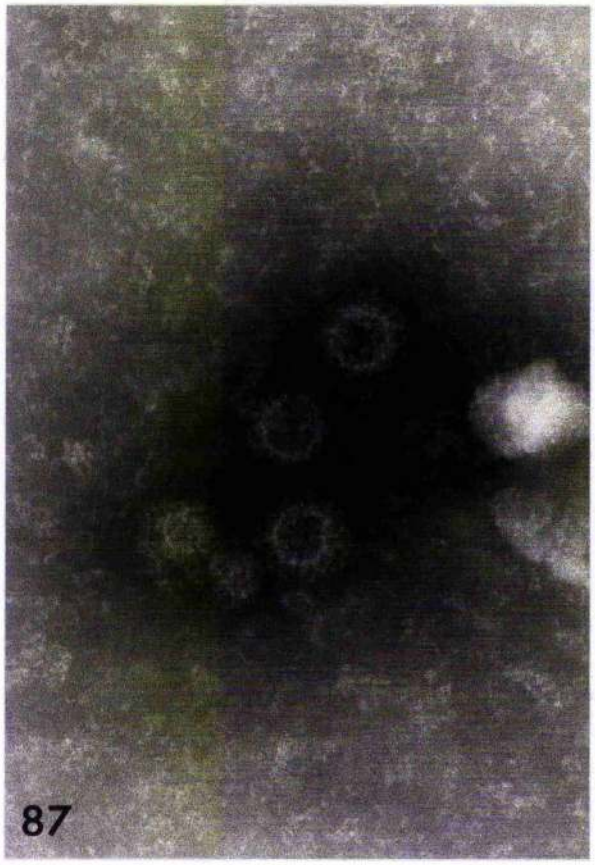
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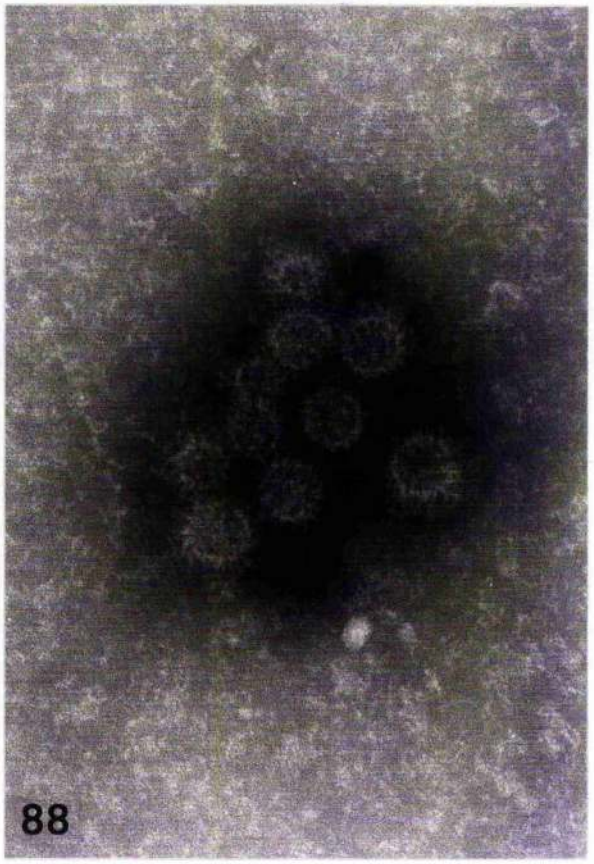

88

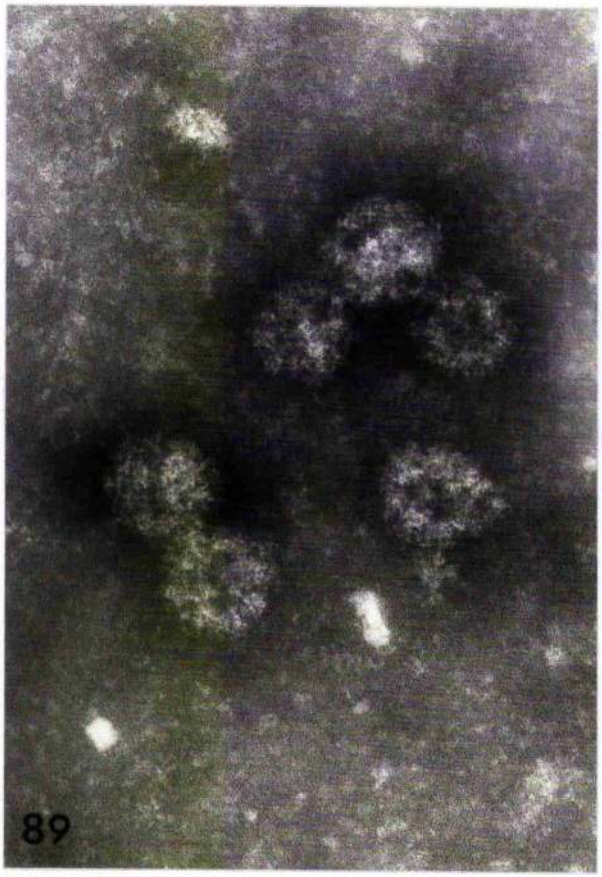
Figures 89 to 92 illustrate some of the non-papovavirus particles that have been observed.

Fig 89. ACG: control grid (without GAB IgG) + CK14V. Although these large, indistinct particles are similar in size to polyomavirions surrounded by a particularly dense antibody coat, evidence of polyomavirus structure could not be found in such particles. x 200,000.

Fig 90. ACG: grid treated with GAB IgG, 1/100 + CK14V. Two chains of elongated, roughly elliptical particles are shown. x 200,000.

Fig 91. High speed pellet of culture fluid from CK29/2 cells after incubation for eight weeks; medium contained 2% equine serum. An aggregate of relatively large, spherical particles is illustrated. x 200,000.

Fig 92. High speed pellet of culture fluid from CK40 cells after incubation for 22 weeks; medium contained 2% gamma-irradiated fetal bovine serum. Bacteriophages, some with tails and some without, form a clump. x 200,000.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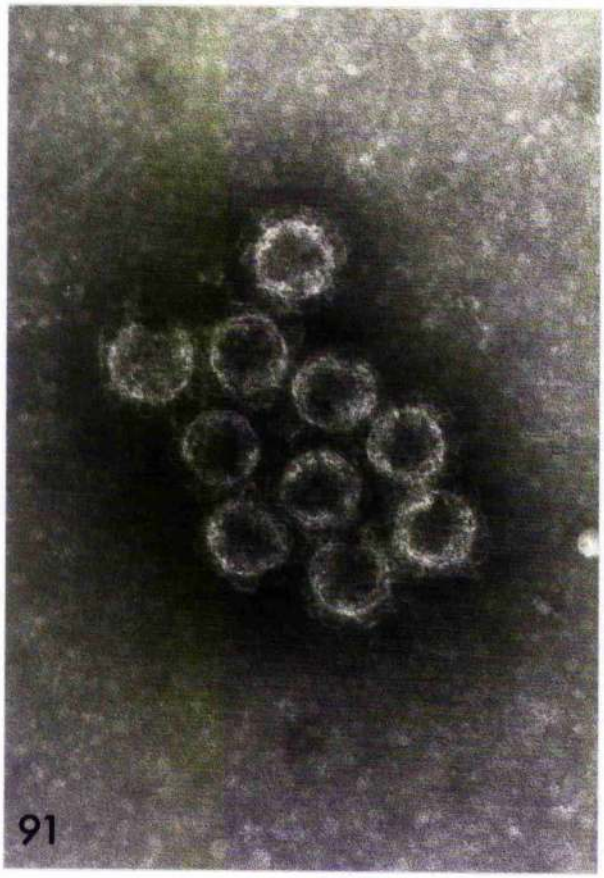
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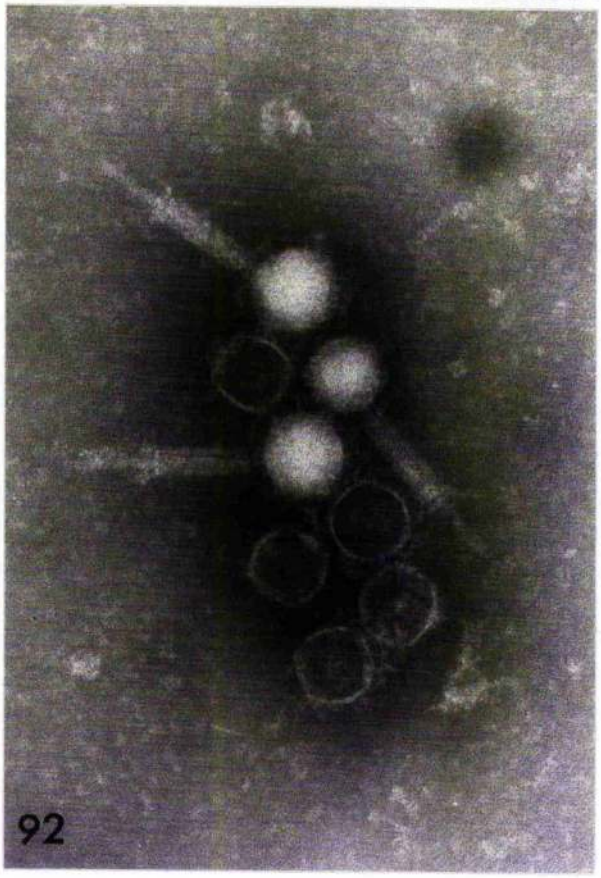

92

Figures 93 to 96 illustrate the association between polyomavirus capsomers and membranes.

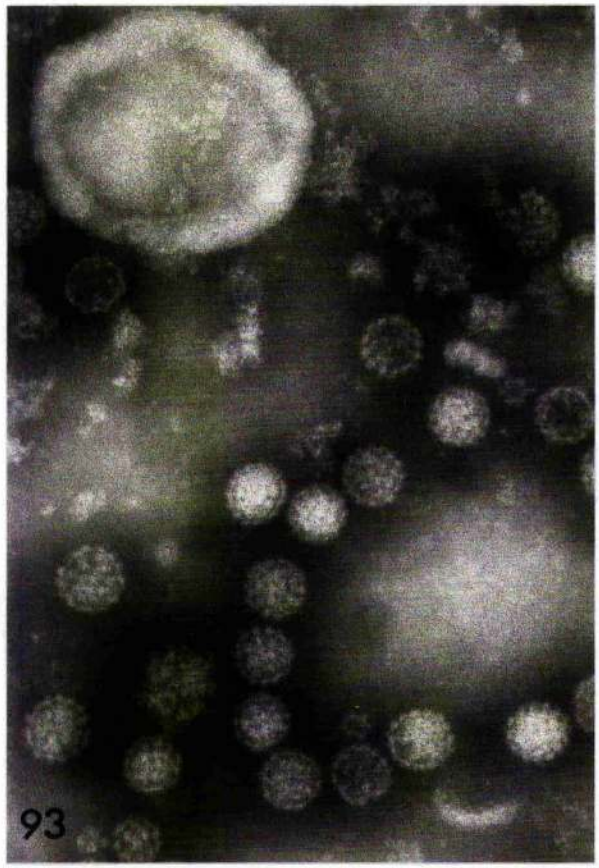
Fig 93. High speed pellet of clarified culture fluid from uninoculated CK28 cells after incubation for 15 weeks; medium contained 2% fetal bovine serum U720201D. Polyomavirus capsomers lie on the surface of a vacuole into which the negative stain has failed to penetrate. Polyomavirions, "mini mini" polyomavirus particles and amorphous substance are also present. x 200,000.

Fig 94. IEM: polyoma virus + mouse polyoma virus antiserum, 1/500. A membranous structure bearing polyomavirus capsomers is attached to polyoma virions by very small amounts of antibody. x 200,000.

Figures 95 and 96 are of a high speed pellet of culture fluid from HEK cells after inoculation with FRK4V; medium contained 2% newborn bovine serum.

Fig 95. Polyomavirus capsomers on this piece of membrane are more easily identified than those in Figure 93. x 200,000.

Fig 96. Capsomers are arranged in regular array on membrane beside which membrane-associated polyomavirions can be seen. x 200,000.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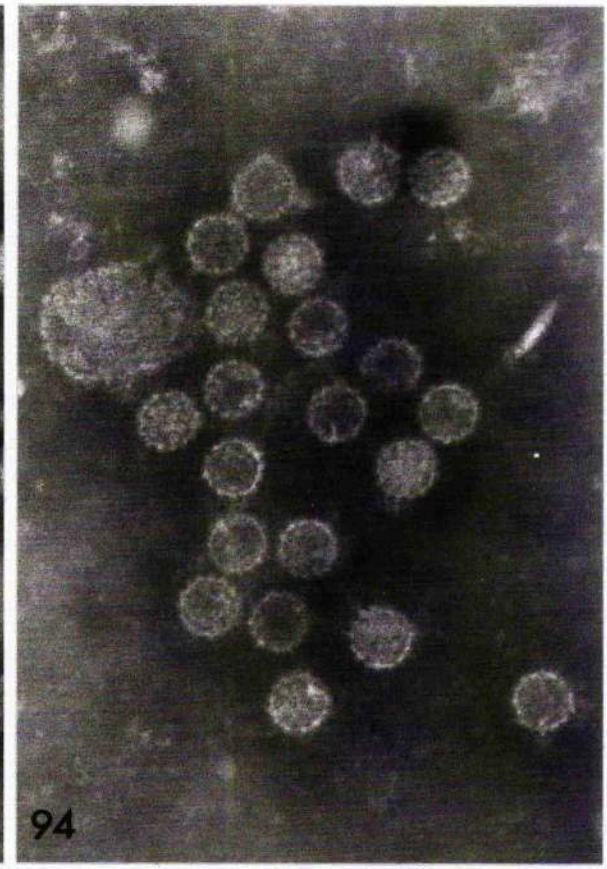
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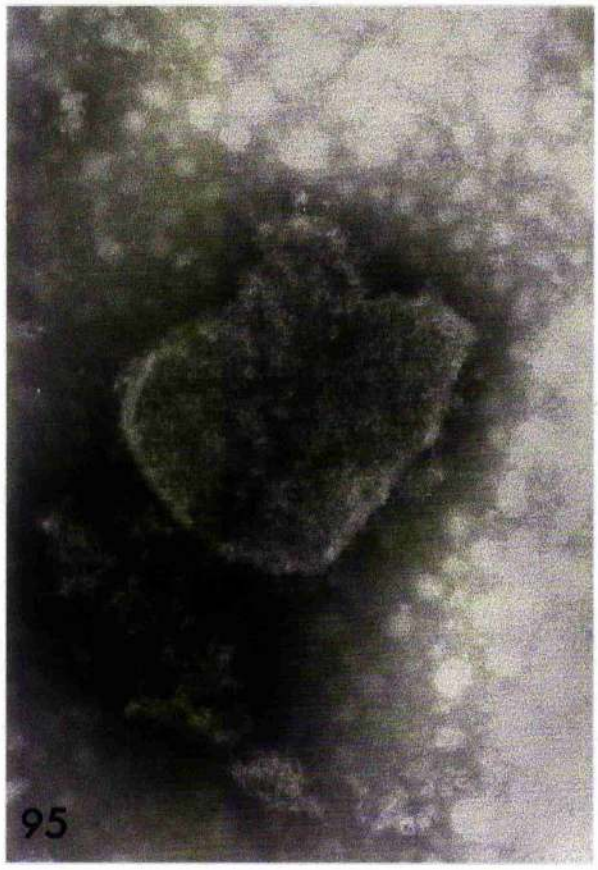
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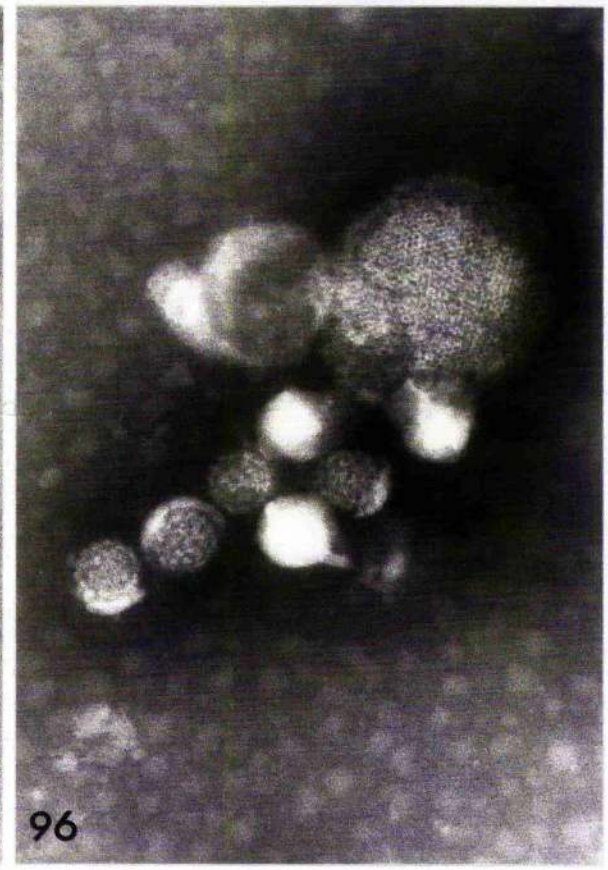

96

Figures 97 to 100 illustrate indistinct structures which may represent the contents of polyomavirus particles.

Figures 97 and 98: high speed pellets of clarified culture fluids from uninoculated CK cells; medium contained 2% fetal bovine serum U720201D.

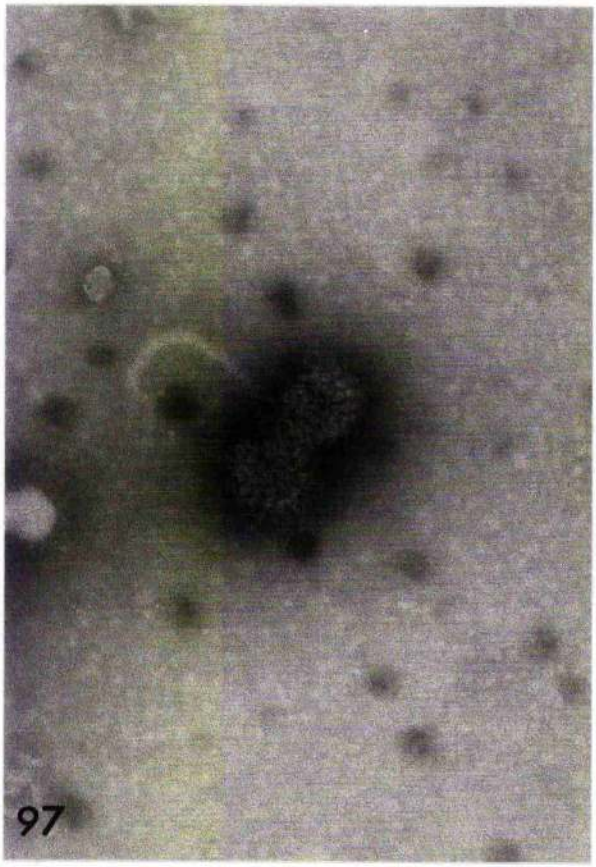
Fig 97. CK14 culture after incubation for 17 weeks. A curved plume of slightly electron-translucent substance lies close to two polyomavirions which are surrounded by dense antibody. x 200,000.

Fig 98. CK21 culture after incubation for 14 weeks. Two trails of slightly electron-translucent substance can be seen and there are free polyomavirus capsomers near one of the damaged polyomavirions. x 200,000.

Figures 99 and 100: high speed pellets of culture fluids from CK29 cells after incubation for 11 weeks.

Fig 99. CK29/1 culture; medium contained 2% gamma-irradiated fetal bovine serum. Indistinct trails are associated with one damaged polyomavirion and a "mini" polyomavirus particle in the middle of the field. x 200,000.

Fig 100. CK29/2 culture; medium contained 2% equine serum. Plumes of slightly electron-translucent substance appear to be escaping from the two damaged polyomavirions at the top of the Figure. Fine strands or amorphous substance connect three of the remaining polyomavirus particles.
x 200,000.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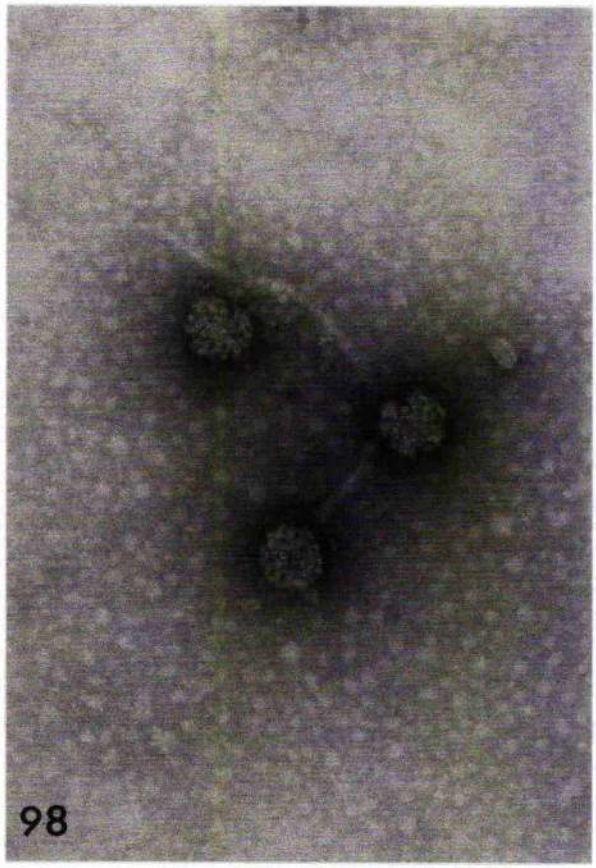
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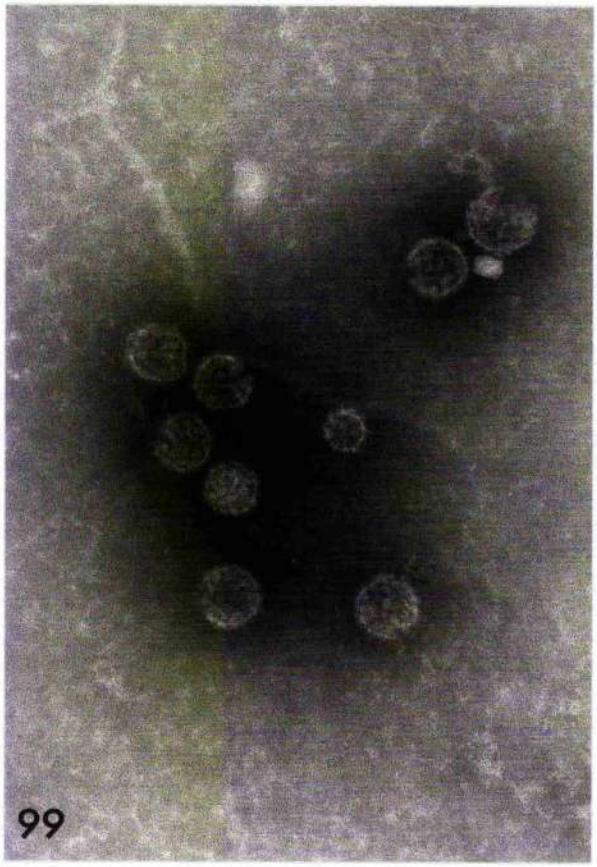
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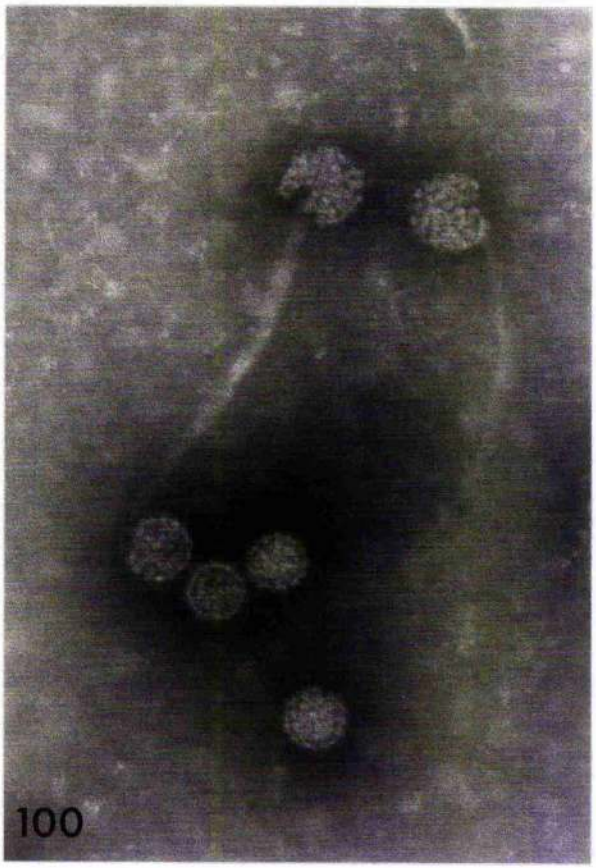

100

Figures 101 to 104 illustrate the diverse numbers and appearance of the small, roundish, featureless particles frequently found associated with polyomavirions.

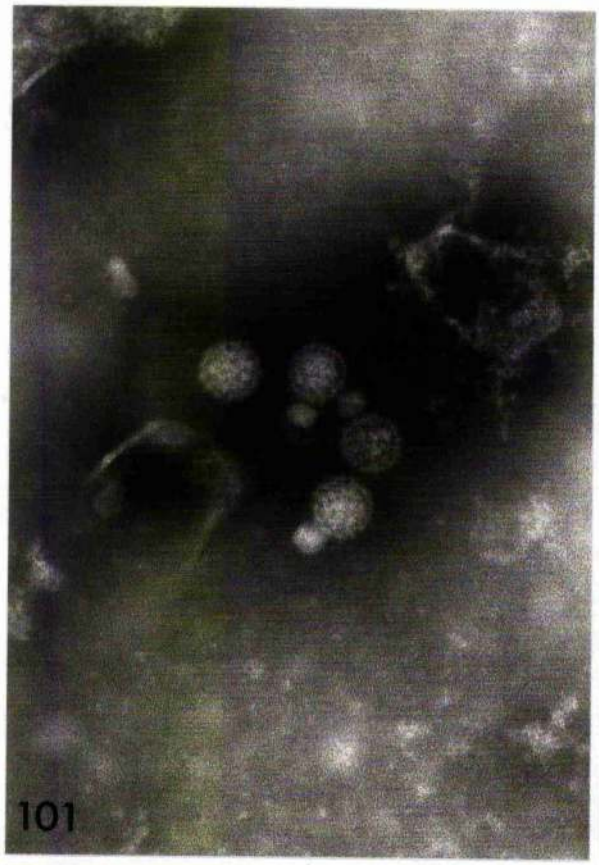
Fig 101. Pooled preparation of spontaneously detached cells from culture fluids from several batches of uninoculated FRhK-4 cells. Three small, round or oval, featureless particles are attached to polyomavirions. x 200,000.

Fig 102. High speed pellet of culture fluid from HEK cells ten days after inoculation with FRK4V; medium contained 2% newborn bovine serum. Four small, roundish particles are included in this clump of polyomavirus particles. x 200,000.

Figures 103 and 104 are of high speed pellets of clarified culture fluids.

Fig 103. Uninoculated FRhK-6 cells; medium contained 2% fetal bovine serum. Three polyomavirions and several small, round particles, one of which is considerably larger than the others, are attached to one another. x 200,000.

Fig 104. Uninoculated CK19 cells after incubation for 16 weeks; medium contained 2% fetal bovine serum U720201D. Two polyomavirions are encircled by small, round or oval particles. x 200,000.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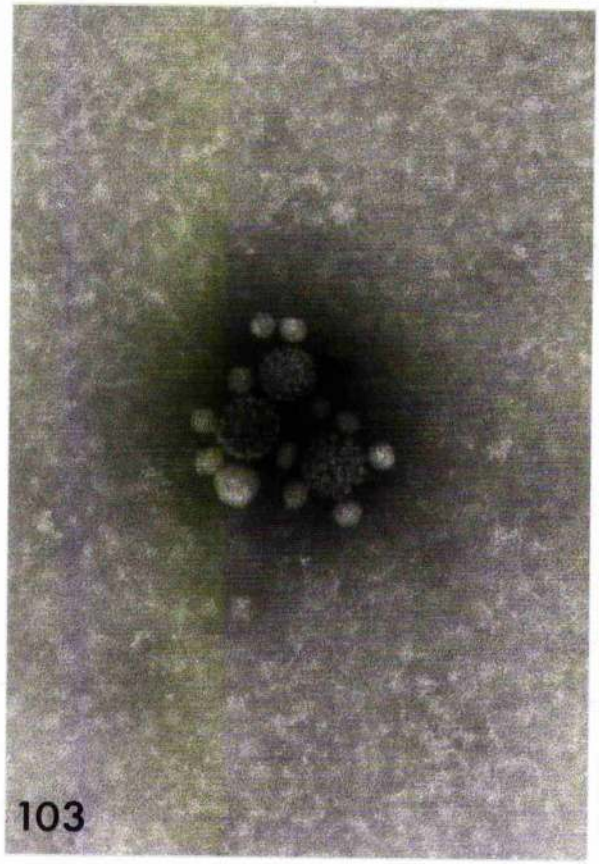
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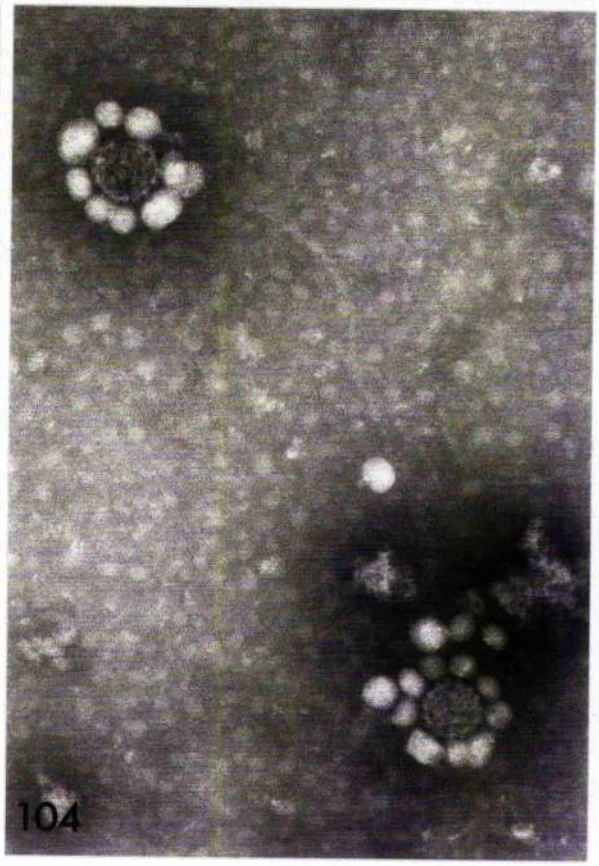

104

Figures 105 to 108 show further examples of small, roundish particles.

Figures 105 and 106 are of the culture fluid from uninoculated CK27 cells after incubation for 18 weeks; medium contained 2% fetal bovine serum U720201D. High speed pellet of culture fluid (Fig 105) and spontaneously detached cells (Fig 106).

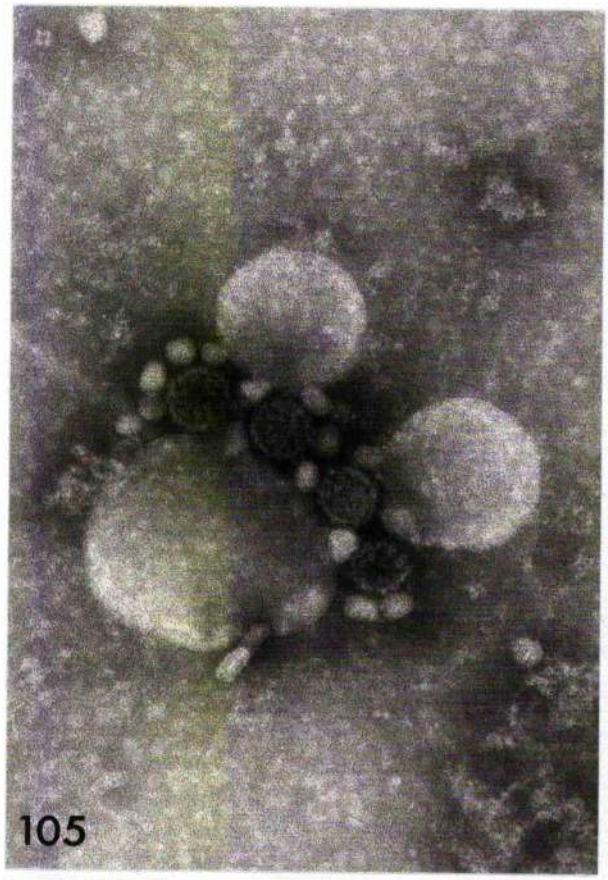
Fig 105. Four polyomavirions are attached to three vacuoles and several small, round particles. Some of the small, round particles might be budding from, or emerging through membranes. x 200,000.

Fig 106. Several small, round particles are attached to this long polyomavirus filament. x 200,000.

Figures 107 and 108 are of high speed pellets of clarified culture fluids from uninoculated CK cells; media contained 2% fetal bovine serum U720201D.

Fig 107. CK22 culture after incubation for 21 weeks. Two small, round particles, one of which appears to have emerged from a damaged polyomavirion, are present. x 200,000.

Fig 108. CK27 culture after incubation for 18 weeks. A small, round particle seems to be escaping from a damaged polyomavirion. x 200,000.



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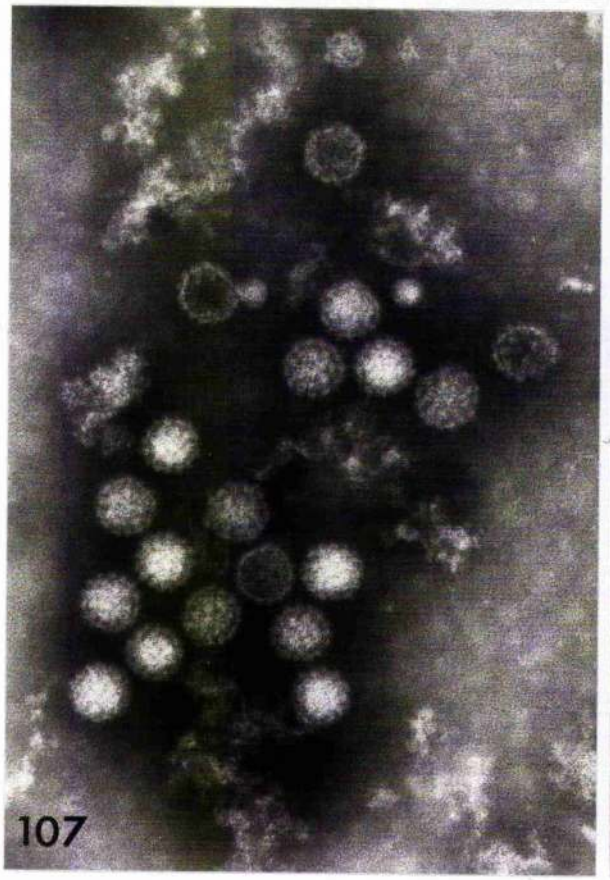

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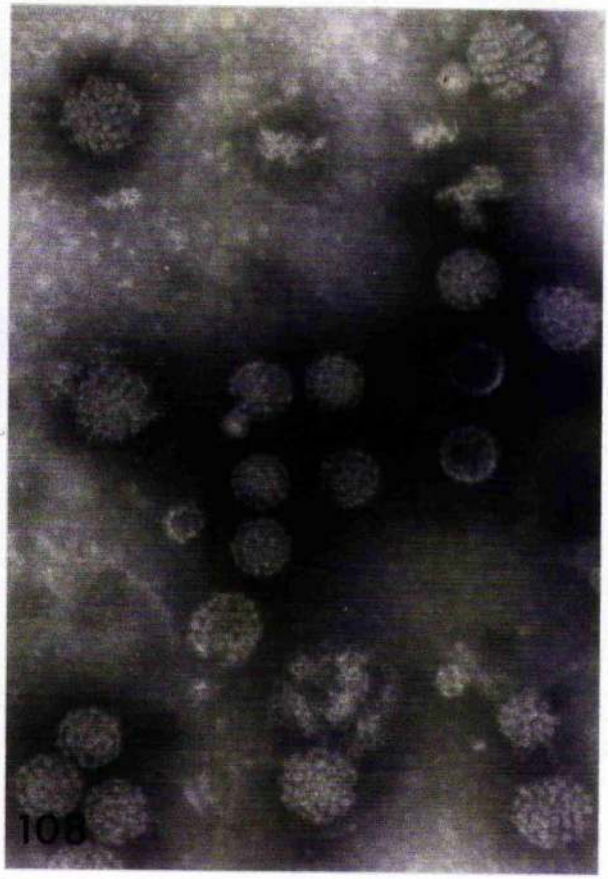


108

Figures 109 to 112 illustrate the association between polyomavirus particles and amorphous substance.

Figures 109 and 110 are of a high speed pellet of culture fluid from uninoculated CK12 cells after incubation for 21 weeks; medium contained 2% fetal bovine serum U720201D.

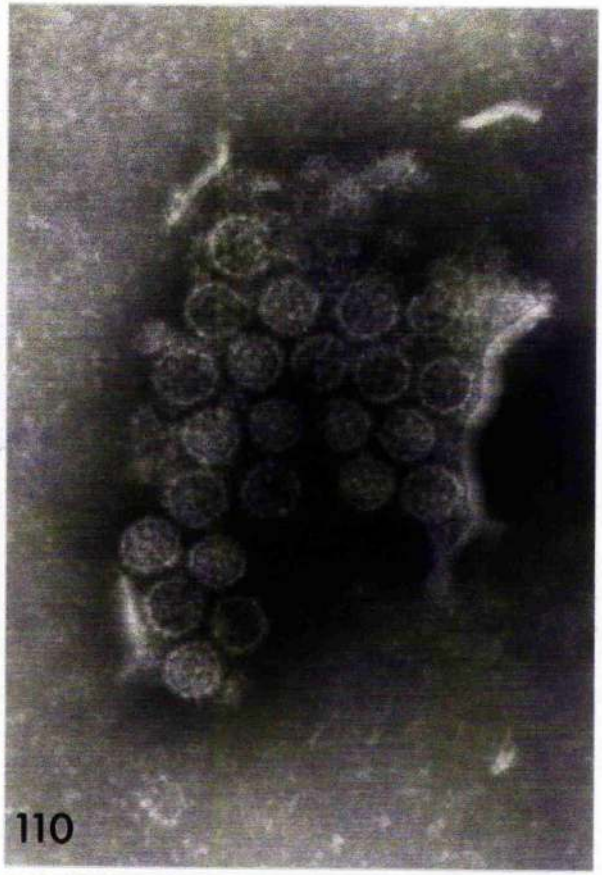
Fig 109. Amorphous substance surrounds and appears to hold together most of the polyomavirus particles in this clump. x 200,000.

Fig 110. Fragments of membrane can be seen around this aggregate of polyomavirions and amorphous substance. x 200,000.

Fig 111. Spontaneously detached cells from culture fluid of CK17 cells 59 days after inoculation with FRK6V; medium contained 2% fetal bovine serum. In the upper part of the field, seven polyomavirions embedded in amorphous substance are almost completely surrounded by fringed membrane. x 200,000.

Fig 112. High speed pellet of culture fluid from Vero cells inoculated with SV40; medium contained 2% newborn bovine serum. The faint outline of virus particles can be identified (arrows) within partially membrane-bound amorphous substance, from the top right of which polyomavirions appear to be escaping. x 200,000.

109



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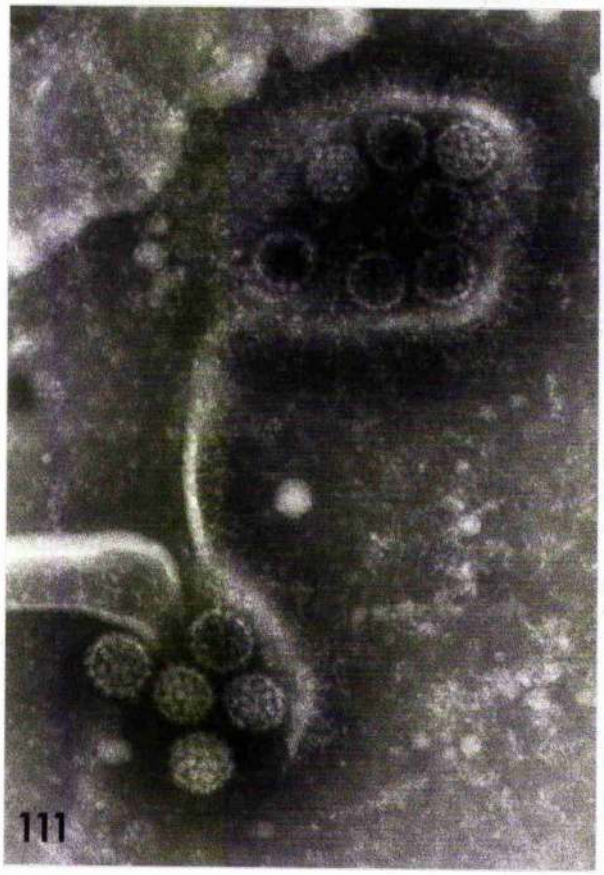

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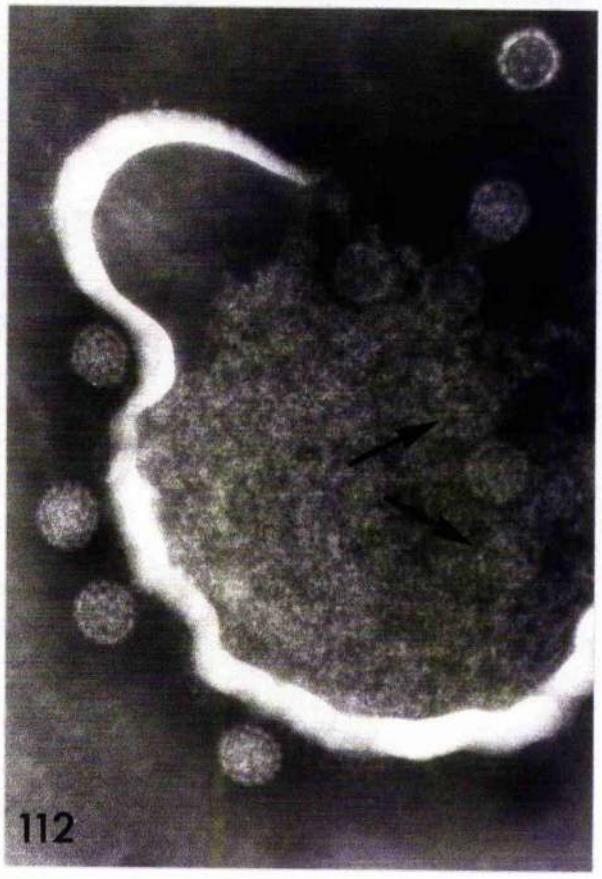
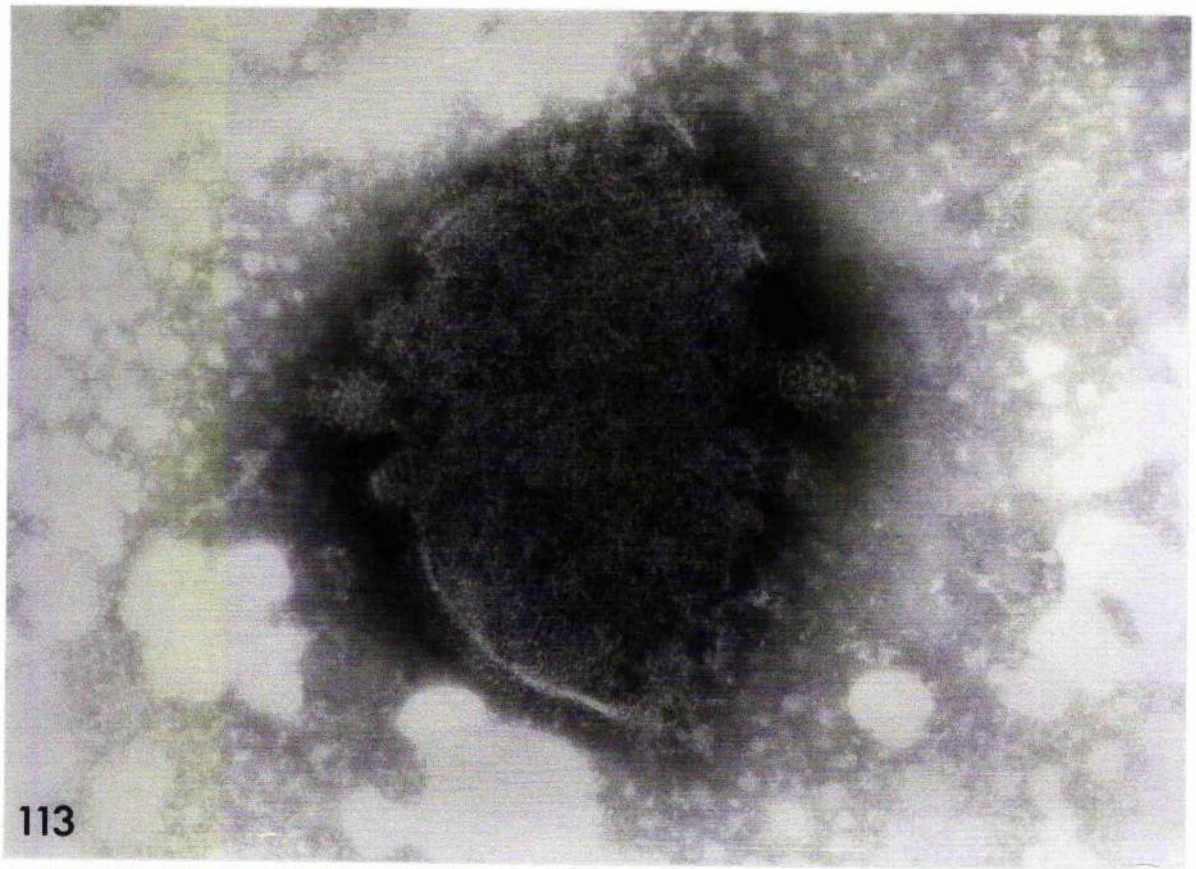


112

Figures 113 and 114 show further examples of papovavirus particles associated with amorphous substance.

Fig 113. High speed pellet of culture fluid from HEK cells 28 days after inoculation with FRK4V; medium contained 2% newborn bovine serum 17/8. Fringed membrane partially surrounds this area of amorphous substance from which three polyomavirions seem to be emerging. x 200,000.

Fig 114. Preparation of human papillomavirus partially purified from plantar wart. Papillomavirions (arrows) appear to be forming within a large area of amorphous substance. x 200,000.



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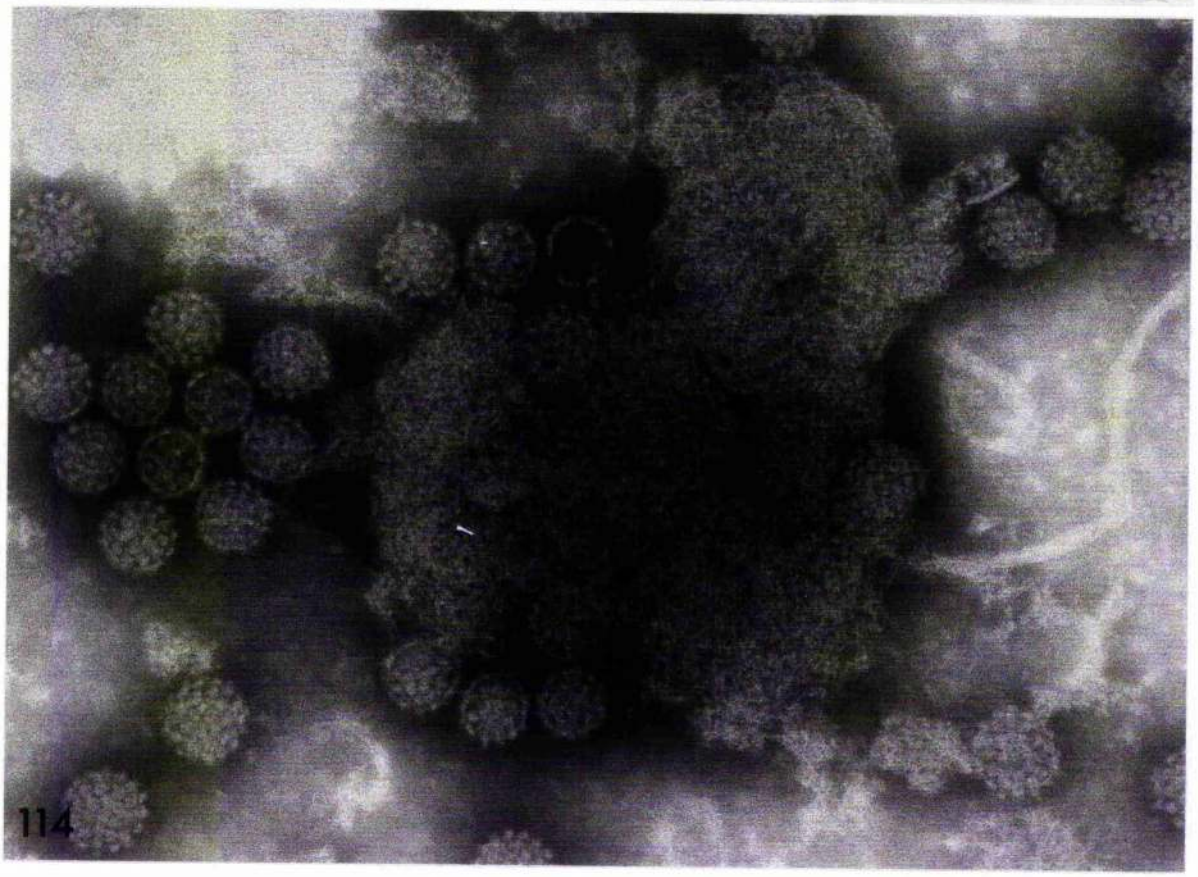

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